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(54) Title: DIAGNOSIS OF ENDOMETRIAL PRECANCERS

(57) Abstract: The invention disclosed herein describes novel methods for diagnosing endometrial precancers by measuring PTEN expression and offers an immunohistochemical biomarker for premalignant disease.

DIAGNOSIS OF ENDOMETRIAL PRECANCERS

Field of the Invention

The invention relates to immunological methods for diagnosis of conditions
5 characterized by abnormal levels of PTEN protein.

Background of the Invention

PTEN tumor suppressor gene mutations are the most frequent genetic lesion in the
highest incidence female genital tract tumor, endometrioid endometrial adenocarcinoma. The
10 role of PTEN in mediating risk conferred by an abnormal hormonal environment and
endometrial hyperplasias is uncertain, because of inadequate reagents for protein expression
studies *in situ* and controversy in precancer diagnosis.

Somatic mutation or deletion of the PTEN tumor suppressor gene has been reported in
approximately 40% (1;2) and 40 (3)-76% (4), respectively, of endometrial adenocarcinomas.
15 Further evidence for PTEN function within the female reproductive tract is evident in Pten
knockout mice that develop complex proliferative endometrial lesions (5). In humans,
familial inheritance of mutant PTEN alleles in Cowden syndrome causes multi-organ
development of benign hamartomatous and malignant epithelial tumors (6-8), including an
elevated incidence of endometrial adenocarcinoma.

20 A particular variant of endometrial cancer contributes the bulk of PTEN mutations.
These are endometrioid endometrial adenocarcinomas (1;2), the most common type [80%
(9;10)] of endometrial cancer in the United States, readily discriminated by routine
histopathology from "non-endometrioid" tumors such as papillary serous and clear cell
adenocarcinomas, which also occur at this site. Risk for endometrioid endometrial
25 adenocarcinomas increases in subjects with high estrogen levels unopposed by progestins
(11), and appearance of a physically distinctive precancerous lesion (12). Interaction between
genetic and hormonal events during the premalignant phases of endometrial tumorigenesis is
expected, yet has never been precisely elucidated.

Inaccessibility of premalignant tissues, controversy in their diagnosis, and paucity of
30 high-yield candidate genes are longstanding barriers to productive exploration of endometrial
precancer biology. PCR methods have improved the analytical repertoire suited to physically
small precancers, including detailed mutational (13), clonal (14), and even lineage
reconstruction (15) analysis. However, accurate diagnosis of the precancers themselves,

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typically termed "hyperplasias" in the widely used World Health Organization nomenclature (16), has been difficult to standardize (17), and even when criteria are agreed upon, reproducibility (18) is suboptimal. Previous reports of PTEN mutation in putative
5 endometrial precancers have used subjective diagnostic criteria (19-21).

Thus, there presently is a need for objective, reproducible, and sensitive methods for diagnosis of endometrial precancers.

Summary of the Invention

10 Loss of PTEN function by mutational or other mechanisms is an early and progressive event in endometrial tumorigenesis that may occur in response to known endocrine risk factors, and offers an immunohistochemical biomarker for premalignant disease. Unexpectedly, it has been discovered that individual PTEN-negative glands in endometria (particularly in subjects exposed to unopposed estrogen) are distinguishable by
15 immunohistochemistry using PTEN antibodies or antigen-binding fragments thereof, and that these glands are the earliest recognizable stage of endometrial carcinogenesis, followed by proliferation into dense clusters that form discrete premalignant lesions. Accordingly, the invention provides improved methods and compositions for diagnosing endometrial precancers. The methods of the invention also may be used for identifying individuals at risk
20 for endometrial cancer, or individuals at risk for the recurrence of endometrial cancer after treatment. The methods of the invention may also be used for identifying pharmaceutical candidate compounds active in the onset, progression, or regression of endometrial cancer or precancer.

According to one aspect of the invention, methods for determining the likelihood of a
25 group of endometrial cells or an endometrial gland to become cancerous are provided. The methods include performing PTEN antibody or antigen-binding fragment thereof immunohistochemistry on a group of endometrial cells or one or more endometrial glands and determining the binding of the PTEN antibody or antigen-binding fragment thereof to the group of endometrial cells or glands. A reduced amount of PTEN antibody or antigen-
30 binding fragment thereof bound to the group of endometrial cells or glands relative to a control group of cells indicates that the group of endometrial cells or glands has an increased likelihood of becoming cancerous.

In preferred embodiments, the PTEN antibody or antigen-binding fragment thereof is

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6H2.1 antibody or antigen-binding fragment thereof.

In certain embodiments, the group of endometrial cells or the one or more endometrial glands and the control group of cells are present in a tissue sample, such as a tissue biopsy. In other embodiments the control group of cells and the group of endometrial cells or glands are the same cell type. In certain preferred embodiments, the amount of binding of the PTEN
5 antibody or antigen-binding fragment thereof to the group of endometrial cells or glands is 50% or less of the binding of the PTEN antibody or antigen-binding fragment thereof to the control group of cells.

In still other aspects of the invention, the methods also include determining the size of
10 a group of endometrial cells or one or more endometrial glands which have reduced PTEN expression. The size of the cells or glands can be measured in control tissues or in a control biopsy of the same subject to establish a baseline size or average size for the cells or glands. Likewise, PTEN expression can be measured in control tissues or in control biopsies of the same subject. In these embodiments, an increased size of the group of endometrial cells or
15 the glands relative to a control group of cells or glands indicates that the group of endometrial cells or glands has an increased likelihood of becoming cancerous.

In some embodiments of the foregoing methods, the group of endometrial cells or glands are obtained from a subject suspected of having endometrial cancer, or from a subject having or suspected of having elevated unopposed estrogen levels. In still other
20 embodiments, the subject is receiving or has received unopposed estrogen treatment.

According to another aspect of the invention, methods for determining regression, progression or onset of a condition characterized by abnormal levels of PTEN protein are provided. The methods include obtaining a level of the amount of PTEN from a sample obtained from a subject and comparing the level to a control as a determination of regression,
25 progression or onset of the condition. In preferred embodiments of these methods, the PTEN antibody is 6H2.1. In certain embodiments, the subject is undergoing drug therapy for a condition characterized by abnormal levels of PTEN protein.

According to still another aspect of the invention, methods for monitoring the progression of endometrial precancers are provided. The methods include determining the
30 expression of PTEN in endometrial cells or glands by PTEN antibody or antigen-binding fragment thereof immunohistochemistry of an endometrial tissue sample obtained at a first time, determining the expression of PTEN in endometrial cells or glands by PTEN antibody or antigen-binding fragment thereof immunohistochemistry of an endometrial tissue sample

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obtained at a second time, and comparing the expression of PTEN in the endometrial cells or glands at the first time and the second time. Reduced expression of PTEN at the second time relative to the first time indicates progression of endometrial precancers to a cancerous stage. In preferred embodiments the PTEN antibody is 6H2.1.

5 In some embodiments, the methods also include determining the size of groups of endometrial cells or glands which have reduced PTEN expression in the endometrial tissue sample obtained at the first time and the endometrial tissue sample obtained at the second time, and comparing the size of the groups of endometrial cells or the glands which have reduced PTEN expression at the first time and the second time. Increased size of the groups
10 of endometrial cells or the glands which have reduced PTEN expression at the second time relative to the first time indicates progression of endometrial precancers to a cancerous stage.

In certain embodiments, the endometrial tissue samples are obtained from a subject suspected of having endometrial cancer or from a subject having or suspected of having elevated unopposed estrogen levels. In other embodiments, the subject is receiving or has
15 received unopposed estrogen treatment or the subject is undergoing drug therapy for endometrial precancer or endometrial cancer.

Similar methods are useful for determining regression of endometrial cancers or precancers, such as following therapeutic invention. Thus, in another aspect of the invention, methods for monitoring the regression of endometrial precancers are provided. The methods
20 include the steps and materials recited above for determination of progression of endometrial precancers. In the analysis of the results, increased expression of PTEN at the second time relative to the first time indicates regression of endometrial precancers from a cancerous stage, and decreased size of the groups of endometrial cells or the glands which have increased PTEN expression at the second time relative to the first time indicates regression of
25 endometrial precancers from a cancerous stage.

In a further aspect of the invention, methods of selecting a treatment for endometrial precancer or endometrial cancer in a subject are provided. The methods include obtaining a level of PTEN expression from an endometrial tissue sample obtained from the subject by immunohistochemical analysis, and selecting the treatment for endometrial precancer or
30 endometrial cancer in the subject based at least in part on the level obtained. These methods also can be used for evaluating a treatment, e.g., to determine its effectiveness.

In certain embodiments, the subject is already receiving drug therapy for endometrial precancer or endometrial cancer. In preferred embodiments, the immunohistochemical

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analysis is performed using 6H2.1 clone PTEN-reactive monoclonal antibody.

In still another aspect of the invention, kits are provided for identifying endometrial precancer cells in an endometrial tissue sample. The kits include a container containing a PTEN antibody or antigen-binding fragment thereof, a second container containing a
5 detectable compound for detecting the presence of the PTEN antibody or antigen-binding fragment thereof in a sample and instructions for binding the PTEN antibody or antigen-binding fragment thereof to an endometrial tissue sample and for measuring the amount of PTEN antibody or antigen-binding fragment thereof bound to the endometrial tissue sample using the detectable compound. The detectable compounds include, for example, enzyme
10 substrates, detectably labeled second antibodies, etc.

In some embodiments the kits also include a container containing a second detectable compound for histological determination of the size of groups of cells or glands in the endometrial tissue sample.

Other kits for identifying endometrial precancer cells in an endometrial tissue
15 according to the invention include a container containing a PTEN antibody or antigen-binding fragment thereof conjugated to a detectable compound, a second container containing a second detectable compound for histological determination of the size of groups of cells or glands in the endometrial tissue sample and instructions for binding the PTEN antibody or antigen-binding fragment thereof to an endometrial tissue sample and for measuring the
20 amount of PTEN antibody or antigen-binding fragment thereof bound to the endometrial tissue sample using the detectable compound. The detectable compounds include, for example, radiolabels, fluorescent labels, colorimetric compounds.

According to a further aspect of the invention, methods for diagnosing endometrial precancer in a subject are provided. The methods include: obtaining a biological sample of
25 endometrial tissue or cells from a subject, contacting the sample with an endometrial cell marker that specifically binds to endometrial cells, contacting the sample with an antibody or antigen-binding fragment thereof that specifically binds PTEN, determining specific binding between the antibody or antigen-binding fragment thereof and PTEN in the sample, and determining the specific binding between the endometrial cell marker, and agents in the
30 sample. In some embodiments, the determination of specific binding of the antibody or antigen-binding fragment thereof and the specific binding of the endometrial cell marker in the sample, are compared to the specific binding of the antibody or antigen-binding fragment thereof and the specific binding of the endometrial cell marker in a control group of cells as a

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diagnosis for endometrial precancer in the subject.

In some embodiments, the antibody or antigen-binding fragment thereof is 6H2.1 antibody or an antigen-binding fragment thereof. In some embodiments the endometrial cell marker is selected from the group consisting of: antibodies and antigen-binding fragments thereof, and ligands. In preferred embodiments, the endometrial cell marker comprises an anti-estrogen receptor antibody or an anti-progesterone receptor antibody. More preferably, the anti-estrogen receptor antibody is ER-ID5 and/or the anti-progesterone receptor antibody is IA6. In some embodiments, the endometrial cell marker comprises estrogen or progesterone. In some embodiments, the sample is menstrual fluid.

In some embodiments, the subject is not suspected of having endometrial cancer, and in other embodiments, the subject is not suspected of having endometrial precancer. In some embodiments, the sample is obtained from a subject having or suspecting of having elevated unopposed estrogen levels and in still other embodiments, the sample is obtained from a subject receiving or having received unopposed estrogen treatment.

According to another aspect of the invention, methods for diagnosing endometrial precancer in a subject are provided. The methods include obtaining a biological sample of endometrial tissue or cells from a subject, isolating the endometrial tissue or cells from the sample, contacting the isolated endometrial tissue or cells with an antibody or antigen-binding fragment thereof that specifically binds PTEN, determining specific binding between the antibody or antigen-binding fragment thereof and PTEN in the isolated tissue or cell sample as a diagnosis for endometrial precancer in the subject. Preferred embodiments include comparing the level of specific binding between the antibody or antigen-binding fragment thereof and PTEN in the isolated tissue or cell sample, and the level of specific binding between the antibody or antigen-binding fragment thereof and PTEN in a matched control tissue or cell sample, as a diagnosis for endometrial precancer in the subject.

In preferred embodiments, the antibody or antigen-binding fragment thereof is 6H2.1 antibody or an antigen-binding fragment thereof. In some embodiments, the sample is menstrual fluid. In certain embodiments, the subject is not suspected of having endometrial cancer, and in other embodiments, the subject is not suspected of having endometrial precancer. In some embodiments, the sample is obtained from a subject having or suspecting of having elevated unopposed estrogen levels, and in some embodiments, the sample is obtained from a subject receiving or having received unopposed estrogen treatment.

According to another aspect of the invention kits for diagnosing endometrial

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precancer in a subject are provided. The kits include antibodies or antigen-binding fragments thereof that specifically bind PTEN, one or more endometrial cell markers, one or more control molecules, and instructions for the use of the antibodies or antigen-binding fragments thereof, cell markers, and control molecules in the diagnosis of endometrial precancer. In some embodiments, the antibodies or antigen-binding fragments thereof are bound to a substrate. In preferred embodiments, the antibody or antigen-binding fragment thereof is 6H2.1 antibody or an antigen-binding fragment thereof. In certain embodiments, the subject is not suspected of having endometrial cancer, and in other embodiments, the subject is not suspected of having endometrial precancer. In some embodiments, the sample is obtained from a subject having or suspecting of having elevated unopposed estrogen levels, and in some embodiments, the sample is obtained from a subject receiving or having received unopposed estrogen treatment.

According to another aspect of the invention, methods for diagnosing endometrial precancer in a subject are provided. The methods include obtaining a biological sample of endometrial tissue or cells from a subject, determining the level of expression of PTEN in the sample, and determining the level of expression of an endometrial cell-associated molecule in the sample. In a preferred embodiment, the method includes comparing the levels of expression of PTEN and one or more endometrial cell-associated molecules to the level of expression of PTEN and one or more endometrial cell-associated molecules in a control sample. In some embodiments, the endometrial cell-associated molecule is selected from the group consisting of: estrogen receptor polypeptides and progesterone receptor polypeptides. In some embodiments, the levels of PTEN and endometrial cell-associated molecule are determined with nucleic acid amplification methods. In some embodiments, the levels of expression of PTEN and the endometrial cell-associated molecules are determined with an immunoassay. In some embodiments, the sample is a body tissue or bodily fluid. In some preferred embodiments, the sample is menstrual fluid. In some preferred embodiments, the sample is endometrial tissue. In certain embodiments, the subject is not suspected of having endometrial cancer, and in other embodiments, the subject is not suspected of having endometrial precancer. In some embodiments, the sample is obtained from a subject having or suspecting of having elevated unopposed estrogen levels, and in some embodiments, the sample is obtained from a subject receiving or having received unopposed estrogen treatment.

According to another aspect of the invention, kits for the diagnosis of endometrial precancer are provided. The kits include oligonucleotides that selectively amplify a nucleic

acid sequence that encodes PTEN, oligonucleotides useful for amplifying the nucleic acid sequence that encode one or more endometrial cell-associated molecules, and control nucleic acid primers. In some embodiments the endometrial cell-associated molecule is selected from the group consisting of: estrogen receptor polypeptides or progesterone receptor polypeptides.

5 According to another aspect of the invention, methods for evaluating the effect of candidate pharmacological compounds on endometrial precancer cell phenotype are provided. The methods include culturing endometrial tissue or cells, contacting the cultured endometrial tissue or cells with an antibody or antigen fragment thereof that specifically binds to PTEN, determining a first amount of specific binding of the antibody or antigen fragment
10 thereof with the endometrial tissue or cells, and contacting the cultured endometrial tissue or cells with a candidate pharmacological agent. The method also includes contacting the cultured endometrial tissue or cells with the antibody or antigen-binding fragment thereof, determining a second amount of specific binding of the antibody or antigen-binding fragment thereof, with the cultured endometrial tissue or cells, and comparing the first and second
15 amounts of specific binding of the antibody or antigen-binding fragment thereof to the tissue or cells. A change in the second amount of specific binding of the antibody or antigen-binding fragment thereof, relative to the first amount of specific binding of the antibody or antigen-binding fragment thereof, indicates the candidate pharmacological compound alters the level of PTEN, wherein a decrease in the relative amount of PTEN indicates the onset of
20 or progression of an endometrial precancer cell phenotype, and where an increase in the relative amount of PTEN indicates the regression of an endometrial precancer cell phenotype. In preferred embodiments, the antibody or antigen-binding fragment thereof is 6H2.1 antibody or an antigen-binding fragment thereof.

 In some embodiments, the endometrial tissue or cells are not suspected of having
25 endometrial cancer and in some embodiments, the tissue or cells are not suspected of having endometrial precancer. In some embodiments, the tissue or cells are from a body tissue or bodily fluid, and in some embodiments, the cells are from menstrual fluid. In some embodiments, the tissue or cells are endometrial tissue or cells. In some embodiments, the tissue or cells have not been exposed to unopposed estrogen levels.

30 According to yet another aspect of the invention methods for evaluating the effect of candidate pharmacological compounds on endometrial precancer cell phenotype are provided. The methods include culturing two matched samples of endometrial tissue or cells, contacting one of the endometrial tissue or cell cultures with a candidate pharmacological

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agent, contacting each of the endometrial tissue or cell cultures with an antibody or antigen fragment thereof that specifically binds to PTEN, determining the amount of specific binding of the antibody or antigen fragment thereof of each of the endometrial tissue or cell cultures, and comparing the amounts of specific binding of the antibody or antigen-binding fragment thereof to the tissue or cell cultures. A difference in the amount of specific binding of the antibody or antigen-binding fragment thereof in the culture contacted with the candidate pharmacological agent, relative to the amount of specific binding of the antibody or antigen-binding fragment thereof in the culture not contacted with the candidate pharmacological agent, indicates the candidate pharmacological compound alters the level of PTEN. A decrease in the relative amount of PTEN indicates the onset of or progression of an endometrial precancer cell phenotype, and where an increase in the relative amount of PTEN indicates the regression of an endometrial precancer cell phenotype. In preferred embodiments, the antibody or antigen-binding fragment thereof is 6H2.1 antibody or an antigen-binding fragment thereof. In some embodiments, the endometrial tissue or cells are not suspected of having endometrial cancer, and in some embodiments, the tissue or cells are not suspected of having endometrial precancer. In some embodiments, the tissue or cells are from a body tissue or bodily fluid, and in some embodiments the cells are from menstrual fluid. In some embodiments, the tissue or cells are endometrial tissue or cells. In some embodiments, the tissue or cells have not been exposed to unopposed estrogen levels.

The foregoing methods include immunohistochemistry and antibody-based methods but one of ordinary skill in the art would realize that alternative methods for quantifying expression of PTEN would also be useful, including but not limited to: hybridization, selective amplification of nucleic acid molecules encoding PTEN and endometrial cell marker molecules.

The foregoing assay also can be used for assessing exposure to environmental estrogens, such as pesticides.

These and other aspects of the invention will be described in further detail in connection with the detailed description of the invention.

Brief Description of the Drawings

Figure 1 is a graph of data from thirty nine non-malignant tissues that were classified as precancers or benign based upon computerized morphometric D-Score (12;23). Each tissue is plotted against the number of PTEN mutant exons detected. Benign tissues (D-Score > 1,

right) did not have PTEN mutations, whereas 55% of morphologic precancers (D-Score<0, left) had mutation in at least one exon. Symbols slightly randomly jittered to improve visibility of overlapping symbols.

Figure 2 is a digitized photomicrograph of immunohistochemical detection of PTEN protein (brown precipitate) by antibody 6H2.1 for one patient amongst areas of endometrial adenocarcinoma, endometrial precancer, and benign endometrium. Carcinoma (Panel A) is devoid of PTEN staining, but adjacent endometrial stromal cells and vascular endothelium contain cytoplasmic and nuclear PTEN protein. A geographic zone of precancerous glands is devoid of PTEN protein (Panel B, upper left), contrasting with abundant stromal staining throughout, and an adjacent region of normal endometrial glands (Panel B, right lower) which demonstrate nuclear and cytoplasmic PTEN. High-magnification views in Panels C and E show interface between PTEN negative (precancer) and benign (PTEN positive) glands, including one transition within an individual gland (Panel E). Companion hematoxylin and eosin stained sections, Panels D and F. Scale bar is 100 μ m.

Figure 3 is a digitized photomicrograph illustrating scattered PTEN-negative glands interposed among PTEN-expressing glands present an interrupted pattern different from the geographic distribution within monoclonal and readily diagnosed precancers shown in Figure 2. This intermittent pattern was seen at a variety of gland densities ranging from the closely packed architecture characteristic of precancers defined by computerized morphometry (Patient 99-47, Panels A-C) to low densities of a disordered proliferative endometrium (unopposed estrogen effect) (Patient 99-30, Panels D-F). Cytology of PTEN expressing and non-expressing glands may be similar (Panels B-C) or different (Panel E-F). Panels G-H show a persistent estrogen exposed endometrium characterized by cysts, which retains epithelial and stromal PTEN expression. Antibody 6H2.1 immunohistochemistry. Companion hematoxylin and eosin stained sections C,F. Scale bar is 100 μ m.

Figure 4 is a digitized photomicrograph image of PTEN Immunohistochemistry in the endometrium. PTEN-null glands (examples indicated by black arrowhead) appear pale green against dark brown staining of surrounding stroma in proliferative (left, Cases 339, 366), persistent proliferative (center, Cases 624, 496), and EIN (right, Cases 847, 593) endometria from six different patients. Brown PTEN-positive glands (white arrowhead) are present in proliferative and persistent proliferative examples for comparison. Scale bar is 500 μ m.

Figure 5 is a diagram of a box-plot of PTEN-null gland morphometric variables (y axis), x axis arranged from left to right in order if a putative progression sequence of

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proliferative (PE, n=20), persistent proliferative (PPE, n=20) and EIN (n=17) endometria stained for PTEN protein. Volume percentage stroma (VPS) declines steadily because of increasing PTEN-null gland volume (VPNULL). Two elements sequentially contribute to rising VPNULL. The first, in the proliferative to persistent proliferative transition, is enlargement of individual PTEN-null glands (SZNULL). The density of PTEN-null glands (DENNULL) then increases between persistent proliferative and EIN endometria. PTEN expressing glands increase in size (SZPOS) in the proliferative to persistent proliferative transition but rapidly decline in density.

Figure 6 is a digitized image (part a) of a single-plex PCR and denaturing gradient gel electrophoresis of the exon 5 of PTEN gene. DNA from PTEN-null glands (NULL) have aberrantly migrating species (arrowheads) compared to PTEN expressing (POS) glands. Bidirectional (for, forward; rev, reverse). The sequencing traces in part b illustrate direct sequence confirmation of mutations. PTEN immunohistochemistry of these two proliferative endometria (#366 and #399) is shown in Figure 4, left-hand boxes.

Detailed Description of the Invention

The invention disclosed herein describes novel methods for diagnosing endometrial precancers and offers an immunohistochemical biomarker for premalignant disease. Unexpectedly, it has been discovered that individual PTEN-negative glands in endometria (particularly those exposed to unopposed estrogen) are distinguishable by immunohistochemistry using PTEN antibodies or antigen-binding fragment thereof, and that these PTEN-negative glands are the earliest recognizable stage of endometrial carcinogenesis, followed by proliferation into dense clusters that form discrete premalignant lesions. Accordingly, the invention provides improved methods and compositions for diagnosing onset, progression, and regression of endometrial precancers.

There are several aspects of the invention that are unexpected. First, it is unexpected that the PTEN marker would be informative at such an early stage of endometrial precancers. For example, in subjects with high estrogen levels unopposed by progesterone, which can happen naturally such as in polycystic ovarian disease (endogenous unopposed estrogen), or pharmacologically such as in estrogen supplementation (exogenous unopposed estrogen), individual endometrial glands have no expression or significantly reduced expression of PTEN, and this identifies them as having altered function, i.e., as premalignant lesions.

Second, it is unexpected that one can track the clinically significant disease over time.

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Clinically significant disease is determined, in part, by the size of the endometrial gland lesion. Using the methods of the invention, one can establish the average size of the precancerous lesions (which differs from those which are not precancerous) and further, by applying PTEN immunohistochemistry, one can detect those lesions that have reduced PTEN expression. Other detection methods of PTEN expression, such as DNA analysis (e.g., PCR or mutational analysis) do not provide information with respect to the size of the lesion or the PTEN expression in specific glands in the tissue.

Third, one can identify the lesions that are precancerous at a very small size. This type of analysis cannot be done effectively using histology alone. In accordance with the invention, one can observe not only the absolute size of the glands, but also which glands have PTEN expression turned off, and thus have a more robust predictor of endometrial precancer.

Fourth, detection of endometrial precancers as a multi-focal or multi-centric disease is also new and unexpected. Cancer typically is thought of as a disease having a single focus; that is, a single cell becomes cancerous, proliferates, and a tumor forms at that locus. The present observations show that endometrial precancers can be formed in several locations simultaneously as several glands can be shown to have reduced PTEN expression and increased size. Without wishing to be bound by any particular theory, it is believed that the initiation event in endometrial precancers, i.e., loss of PTEN expression in a cell, causes the cell to proliferate abnormally. This initiation event unexpectedly has been shown to be a high frequency event in that it occurs in multiple places in the endometrial tissue. To become an endometrial cancer, the lesions must persist and become physically larger. Therefore, an unexpected part of the results is that there is a quantitative threshold event (as opposed to a qualitative threshold event) in that one can observe an abundance of locations having reduced PTEN expression, at least some of which are likely to progress to endometrial cancer.

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cancerous or precancerous is the result of a secondary event, such as exposure to unopposed estrogen or other drug. This surprising finding identifies the premenopausal, non diseased, endometrial tissues as an early carcinogenesis model for endometrial precancer and endometrial cancer. The novel and unexpected carcinogenesis model, utilizing normal
5 (cancer-free) tissue or tissue identified as precancerous or cancerous, may be used to examine the onset, progression or recession of endometrial precancer and endometrial cancer. Such models may also be useful for assessing the effect of candidate pharmacological agents in the onset or treatment of endometrial cancer or precancer.

Seventh, it is new and unexpected that shed cells from the endometrium may be used
10 in the diagnosis of endometrial precancer or endometrial cancer. Such cells may be collected, for example, from menstrual fluid and the expression of PTEN assessed either with immunohistochemical assays or with alternate methods such as selective nucleic acid amplification.

There are several uses for the invention. First, one can use the PTEN
15 immunohistochemistry as an "index diagnostic" to assign risk based on the size of the lesion and the absence of PTEN expression. Therefore, based on these parameters, one can determine whether or not different therapeutic modalities (i.e., chemotherapy, radiation therapy, surgery) should be used. Second, one can use the methods for monitoring progression of endometrial precancers into a cancerous phenotype. By using serial sampling
20 (i.e., biopsy) of the endometrial tissue and observing the size of the lesions and the state of PTEN expression in the lesions (i.e. individual endometrial glands), one can determine whether or not the endometrial precancers are progressing in a way that would indicate whether therapeutic intervention is advised or is successful. Third, one can culture endometrial tissue and test candidate pharmaceutical compounds for effects on PTEN
25 expression.

In the invention, Endometrial Intraepithelial Neoplasia, or "EIN," designates precancers diagnosed using computerized morphometry, and as used herein, "precancers" are synonymous with EIN.

One aspect of the invention is a method to determine the likelihood of a group of
30 endometrial cells or an endometrial gland to become cancerous e.g., for these cells or glands to become precancers or progress to cancerous lesions. The endometrium is the uterine mucous membrane above the level of the internal os. The endometrium consists of a number of subsections such as the endometrial glands, epithelium lining of the endometrial surface,

and the stroma (Novak's Gynecologic and Obstetric Pathology with Clinical and Endocrine Relations, E.R. Novak, and J.D. Woodruff, WB Saunders, Philadelphia, 1979. pp171-172). The invention utilizes an agent, such as an antibody, that specifically binds to PTEN protein to assess levels of PTEN in endometrial tissue and cells. PTEN expression in endometrial
5 cells and tissue may also be assessed using nucleic acid analysis, such as selective amplification, or hybridization methods. A level of PTEN below normal or control levels, indicates an increased likelihood that premalignant endometrial disease is present i.e., that the endometrial cells or tissues are precancers.

According to the present invention, an agent that specifically binds to PTEN is used in
10 diagnosis of endometrial precancers. Preferably agents that bind PTEN are PTEN antibodies or antigen-binding fragments thereof, including polyclonal and monoclonal antibodies, prepared according to conventional methodology. Antibodies and antigen-binding fragments thereof that bind PTEN are useful for determining PTEN levels. Thus, terms such as "PTEN
15 antibody bound to the group of endometrial cells or glands" and "binding of the PTEN antibody to the endometrial cell or gland" means the ability of the antibody to bind to and distinguish PTEN from other proteins. As used herein, the term "antibody" is meant to include antibody or antigen-binding fragment thereof.

Antibodies and antigen-binding fragments thereof that bind PTEN molecules and are useful for determining PTEN levels, include but are not limited to: antibodies or antigen-
20 binding fragments thereof that bind specifically to PTEN and antibodies that bind specifically to fragments of PTEN. Certain antibodies useful in the methods of the invention already are known in the art and include anti-PTEN antibodies, including but not limited to: polyclonal rabbit anti-PTEN MMAC1 (Zymed, PCS Biologicals); PTEN clone A2b1 Hu Ms monoclonal (Chemicon); PTEN N-19 and A2B1 (Santa Cruz Biotechnology); PTEN C terminus
25 polyclonal rabbit anti-PTEN (Zymed); PTEN mouse anti-human monoclonal AB-1 (Calbiochem, Clone 6B1); and PTEN mouse anti-human monoclonal AB-2 (Calbiochem, Clone 1A7). Preferably, the anti-PTEN antibody is 6H2.1 (33).

Also useful in the invention are endometrial cell-associated molecules and the nucleic acids that encode them. Examples of endometrial cell-associated molecules are estrogen
30 receptor polypeptides and progesterone receptor polypeptides. Endometrial cell markers are also useful in this invention and these are molecules that bind to the endometrial cell-associated molecules and include, but are not limited to: ligands such as estrogen and progesterone, and antibodies or antigen-binding fragments thereof that specifically bind to

estrogen receptor polypeptides or progesterone receptor polypeptides.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W. R. (1986) *The Experimental Foundations of Modern Immunology*, Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')₂ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd Fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (Frs), which maintain the tertiary structure of the paratope (see, in general, Clark, W. R. (1986) *The Experimental Foundations of Modern Immunology*, Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. See, e.g., U.S. patents 4,816,567, 5,225,539, 5,585,089, 5,693,762 and 5,859,205.

Fully human monoclonal antibodies also can be prepared by immunizing mice

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transgenic for large portions of human immunoglobulin heavy and light chain loci. Following immunization of these mice (e.g., XenoMouse (Abgenix), HuMAb mice (Medarex/GenPharm)), monoclonal antibodies can be prepared according to standard hybridoma technology. These monoclonal antibodies will have human immunoglobulin amino acid sequences and therefore will not provoke human anti-mouse antibody (HAMA) responses when administered to humans.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')₂, Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric F(ab')₂ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

Thus, the invention involves polypeptides of numerous size and type that bind specifically to PTEN molecules. These polypeptides may be derived also from sources other than antibody technology. For example, such polypeptide binding agents can be provided by degenerate peptide libraries which can be readily prepared in solution, in immobilized form or as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

Phage display can be particularly effective in identifying binding peptides useful according to the invention. Briefly, one prepares a phage library (using e.g. m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array. One then can select phage-bearing inserts which bind to PTEN molecules. This process can be repeated through several cycles of reselection of phage that bind to the PTEN molecules. Repeated rounds lead to enrichment of phage bearing particular sequences. DNA sequences analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the PTEN molecules can be determined. One can repeat the procedure using a biased library containing inserts containing part of all of the

minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the PTEN molecules. Thus, PTEN molecules can be used to screen peptide libraries, including phage display libraries, to identify and select peptide binding partners of the PTEN molecules.

As detailed herein, the foregoing antibodies and other binding molecules may be used for example to isolate and identify PTEN protein. The antibodies may be coupled to specific diagnostic labeling agents for imaging of the protein or fragment thereof. The antibodies may also be used for immunoprecipitation, immunoblotting PTEN using standard methods known to those of ordinary skill in the art.

The immunohistochemistry assays described herein are carried out on samples (specimens) obtained from subjects. As used herein, a subject is a human, non-human primate, cow, horse, pig, sheep, goat, dog, cat, or rodent. In all embodiments, human subjects are preferred. As used herein, samples may be endometrial tissue or cells and may be obtained through standard methods such as biopsy, curettage or from body fluids such as menstrual fluid.

Particularly, important groups of subjects to which the present invention can be applied are premenopausal subjects, subjects suspected not to have endometrial cancer, subjects suspected of having endometrial cancer, and subjects with elevated unopposed estrogen levels. The term "unopposed estrogen" as used herein, means estrogen without progestin (Harrisons, Vol 14, Principles of Internal Medicine, Eds. Fauci, A.S., E. Braunwald, K.J. Isselbacher, J.D. Wilson, J.B. Martin, D.L. Kasper, S.L. Hauser, D.L. Longo, McGraw-Hill, New York, 1999). The term "elevated" as used herein, means levels of unopposed estrogen that exceed normal ranges, which are well known to one of ordinary skill in the medical arts. Elevated estrogen levels may arise from exogenous sources including, but not limited to, administration of estrogen without simultaneous progestin treatment. Elevated unopposed estrogen levels may also arise from endogenous sources such as polycystic ovarian disease.

The assay described herein involves measuring levels of PTEN expression. Levels of PTEN can be determined in a number of ways when carrying out the various methods of the invention. One measurement of the level of PTEN is a measurement of absolute levels of PTEN. This could be expressed, for example, in terms of number of PTEN-positive cells per 100 cells in the tissue sample. Another measurement of the level of PTEN is a measurement of the change in the level of PTEN over time. Still another measurement relates to the number of endometrial glands that express PTEN in a sample. These measurements may be expressed in

an absolute amount or may be expressed in terms of a percentage increase or decrease over time. In one particularly important measurement, the level of PTEN is measured in relation to levels in a control cell or gland sample.

Levels of PTEN are advantageously compared to controls according to the invention.

5 The control may be a predetermined value, which can take a variety of forms. It can be a single cut-off value, such as a median or mean. It can be established based upon comparative groups, such as in groups not having elevated unopposed estrogen levels and groups having elevated unopposed estrogen levels. Another example of comparative groups would be groups having a particular disease, condition or symptoms and groups without the disease, condition or
10 symptoms such as a group with endometrial precancer or endometrial cancer and a group without endometrial precancer or endometrial cancer. Another comparative group would be a group with a family history of a condition such as endometrial cancer and a group without such a family history. The predetermined value can be arranged, for example, where a tested population is divided equally (or unequally) into groups, such as a low-risk group, a medium-
15 risk group and a high-risk group or into quadrants or quintiles, the lowest quadrant or quintile being individuals with the lowest risk or highest amount of PTEN and the highest quadrant or quintile being individuals with the highest risk or lowest amount of PTEN.

Still other controls can be based on other cells or glands within a single endometrial tissue sample. For example, as shown in the Figures, endometrial glands that express PTEN
20 may be located adjacent to endometrial glands that express reduced levels of PTEN. These glands that express PTEN can serve as positive controls for comparison with glands having reduced PTEN antibody staining. Likewise, stromal and other cells in an endometrial tissue sample will express PTEN and can be used as controls.

The predetermined value of a control will depend upon the particular population
25 selected. For example, an apparently healthy population will have a different 'normal' range than will a population which is known to have a condition related to endometrial precancer, endometrial cancer, or elevated unopposed estrogen levels. Accordingly, the predetermined value selected may take into account the category in which an individual falls. Appropriate ranges and categories can be selected with no more than routine experimentation by those of
30 ordinary skill in the art. By "elevated" it is meant high relative to a selected control. Typically the control will be based on apparently healthy normal individuals in an appropriate age bracket.

It will also be understood that the controls according to the invention may be, in addition to predetermined values, samples of materials tested in parallel with the experimental materials.

Examples include samples from control populations or control samples generated through manufacture to be tested in parallel with the experimental samples. As used herein a "matched" control means tissue or cells obtained at the same time from the same subject, for example, parts of a single biopsy, or parts of a single cell sample from the subject.

5 The various assays used to determine the levels of PTEN include: specific binding assays, such as described in the Examples below, using materials which bind specifically to PTEN; gel electrophoresis; and the like. Immunoassays may be used according to the invention including sandwich-type assays, competitive binding assays, one-step direct tests and two-step tests such as described herein. Preferably PTEN levels are determined by nondestructive
10 imaging of PTEN expression. In preferred embodiments, the imaging is real-time imaging and/or permits visualization of PTEN distribution.

 In the methods of the present invention, a labeling agent may be directly or indirectly bound to the PTEN-reactive monoclonal antibody in accordance with any known technique. When the labeling agent is directly bound, the labeling agent may desirably have introduced
15 therein a functional group which is capable of binding to a PTEN-reactive monoclonal antibody. When the labeling agent is indirectly bound, bridging molecules such as avidin-biotin, for example, may be present between the labeling agent and the monoclonal antibody.

 The labeling agent used in the process of the present invention may typically be an enzyme, a chemiluminescent reagent, a fluorescent reagent, and a radioisotope. Typical
20 enzymes are horseradish peroxidase, alkaline phosphatase, β -galactosidase, luciferase, glucose-6-phosphate dehydrogenase (G6PDH), glucose dehydrogenase (GDH), and the like. Typical chemiluminescent reagents are luminol, isoluminol, an acridinium ester, a dioxethan, and the like. Typical fluorescent reagents are fluorescein isothiocyanate, umbelliferone, chelates of a rare earth metal, and the like. Typical isotopes are ^{125}I , ^{14}C and the like. Other
25 labeling agents will be known to one of ordinary skill in the art.

 When the labeling agent employed is an enzyme, a substrate is used for measuring the enzyme activity. The substrate employed is not limited so long as the substrate allows for the enzyme-substrate reaction to be measured as corresponding to the quantity of the enzyme present. For example, when the labeling agent is peroxidase, the substrate employed may be
30 H_2O_2 and 3-3'-diaminobenzidine (which results in the antibody binding site being stained brown) or H_2O_2 and 4-chloro-1-naphthol (resulting in a blue stain), tetramethylbendidine- H_2O_2 , o-phenylenediamine- H_2O_2 , 5-aminosalicylic acid- H_2O_2 , and the like. When the labeling agent is alkaline phosphatase, the substrate employed may be, for example, toluidine

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salt of 5-bromo-4-chloro-3-indolylphosphate. When the enzyme is β -galactosidase, the substrate employed may be, for example, p-nitrophenyl- β -D-galactopyranoside.

In some embodiments a qualitative determination with the naked eye of the quantity of the labeling agent in the specimen may be preferable. In such cases, a preferred measurement system is a colorimetric system wherein the labeled substance includes an enzyme such as peroxidase, alkaline phosphatase, or β -galactosidase as the labeling agent, and wherein an increase in quantity of the enzyme reaction product may be determined by means of a color development visible to the naked eye.

In addition to the immunohistochemical methods of the invention, selective amplification assays such as PCR, may be used to determine the expression levels of PTEN and endometrial cell-associated molecules.

The specimen used in the process of the invention preferably is endometrial tissue or cells collected from the uterine cavity of a subject. The specimen collected from the uterine cavity may be used as collected. Ordinarily, however, the specimen will be treated with reagents appropriate for preparing the specimen for immunohistochemistry. The specimen may be collected by any usual clinical technique, such as biopsy. Another preferred sample or specimen in the process of the invention is cells or tissue collected from menstrual fluid. The cells may be used as collected or may be isolated from non-endometrial cells using standard cell separation procedures known to those of ordinary skill in the art.

The immunohistochemical assays used in the methods of the invention are standard and well known to one of ordinary skill in the art. Examples of such methods are provided below in the Examples. In general, the assay methods include some or all of the following steps. A specimen is prepared by collecting endometrial cells and/or endometrial tissue from the cavity of the uterus, and optionally treating the cells and/or the tissues in accordance with standard histochemical procedures. For example, the tissue sample may be fixed using formalin or other fixatives and further treated to optimize immunohistochemistry. In preferred embodiments, the tissue specimen is embedded in paraffin according to standard histological methods.

A predetermined amount of the specimen is then immobilized on a carrier, which can be, for example, a plastic or glass microscope slide, etc. The immobilized sample is then contacted with a PTEN-reactive antibody (preferably a monoclonal antibody and particularly preferably antibody clone 6H2.1), which optionally is labeled. The tissue sample can be stained using suitable histological counterstain for visualization of tissue (e.g.,

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hematoxylin/eosin [H&E], methyl green) before or after the sample is contacted with the antibody. If the anti-PTEN antibody is not labeled, it may be labeled subsequent to tissue binding by standard methods, such as by binding a labeled secondary antibody, or by contacting the antibody with a detectable reagent (such as an enzyme substrate, or an avidin-
5 tagged detectable compound).

At various times during the methods, the immobilized sample can be washed to remove excess and/or non-specifically bound antibody, stain, detectable compounds, etc., as necessary. Wash solutions and methods for using them will be known to one of ordinary skill in the art.

10 The signal intensity of the labeled reagents bound to the tissue specimen is then measured as a determination of the level of PTEN expression. The size of the endometrial glands or of other groups of cells in the tissue specimen can also be measured and the measurement used in the diagnostic methods of the invention as a further predictor of endometrial precancers. The average size of the endometrial glands or of other groups of cells
15 in the tissue specimen may be measured prior to treatment to establish a baseline size.

As disclosed herein, it is also possible to assess likelihood of endometrial precancer by monitoring changes in the absolute or relative amounts of PTEN over time. For example, as disclosed herein, a decrease in PTEN expression in individual endometrial glands correlates with increasing likelihood of endometrial precancer arising in such glands. Accordingly one can
20 monitor PTEN expression over time to determine if the likelihood of endometrial precancer in a subject is changing. Decreases in relative or absolute PTEN of greater than 1.0% may indicate an abnormality, for example an onset or progression of endometrial precancer or endometrial cancer. Preferably, the decrease in PTEN levels, which indicates an abnormality, is greater than 2.0%, 3.0%, 4.0%, 5.0%, 7.0%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, or more. Increases in
25 amounts of PTEN expressed in endometrial glands over time may indicate a decrease in precancer or endometrial cancer remission or regression.

The invention in another aspect provides a diagnostic method to determine the effectiveness of treatments. The "evaluation of treatment" as used herein, means the comparison of a subject's levels of PTEN measured in samples collected from the subject at
30 different sample times, preferably at least 1 month apart following treatment. The preferred time to obtain the second sample from the subject is at least one month after obtaining the first sample, which means the second sample is obtained at any time following the day of the first sample collection, preferably at least 30, 45, 60 or more days after the time of first sample

collection.

The comparison of levels of PTEN in two or more samples, taken on different days, allows evaluation of disease progression or regression and of the effectiveness of anticancer treatment. The comparison of a subject's levels of PTEN measured in samples obtained on
5 different days provides a measure to determine the effectiveness of any treatment to avoid or eliminate endometrial precancer.

As will be appreciated by those of ordinary skill in the art, the evaluation of the treatment also may be based upon an evaluation of the symptoms or clinical end-points of the associated disease. Thus, the methods of the invention also provide for determining the
10 regression, progression, or onset of a condition which is characterized by reduced levels of PTEN. In some instances, the subjects to which the methods of the invention are applied are already diagnosed as having a particular condition or disease. In other instances, the measurement will represent the diagnosis of the condition or disease. In some instances, the subjects will already be undergoing therapy for precancer or cancer, while in other instances the
15 subjects will be without present therapy for precancer or cancer.

The diagnostic methods of the invention preferably also include a determination of the size of individual endometrial glands or groups of glands or cells in an endometrial tissue sample. The size of the glands or groups of cells can serve as a further diagnostic marker of endometrial precancers. Typically, progression to a cancerous phenotype includes an increase in
20 cellular proliferation manifested as an increased size of a gland or group of cells. The size of a gland or group of cells can be measured by any convenient method, such as determining the maximum linear dimension of a gland or group of cells. The "maximum linear dimension" of a gland or group of cells is the longest straight linear measurement across the gland or group of cells. Sizes of glands or groups of cells which will confirm or contribute to a diagnosis of
25 endometrial precancer can be absolute sizes or relative sizes as compared to control glands or groups of cells. For absolute sizes, a preferred maximum linear dimension of a gland or group of cells is at least one millimeter. More preferably, the maximum linear dimension is at least 2.0, 2.5, or 3.0 millimeters.

PTEN levels in tissues and cells may also be assessed in tissue and cell culture. Such
30 cultured cells and tissues may be from normal subjects or from subjects believed to have endometrial precancer or endometrial cancer. The cultured cells and tissues may serve as a model for carcinogenesis in endometrial cancer and precancer and may also be used to screen candidate pharmacological agents for effects on the onset, progression or regression of

endometrial precancer or endometrial cancer.

Standard methods of cell and tissue culture may be used to culture endometrial cells and tissues. Examples of such methods, although not intended to be limiting, include an explant model of short term culture of intact chunks of endometrial tissues (see: Bersinger N.A., et al.,
5 Early Pregnancy 1995; 1:134-140; Dudley, D.J., et al., Am J. Obstet Gynecol 1992; 167:1774-1780) and the dissociation of stromal and epithelial parts and re-assembly as a co-culture (Arnold, J.T., et al, Hum Reprod 2001; 16(5):836-845; Hopfer, et al., Pathobiology 1994; 62(2):104-108. The invention includes the use of such culture systems for the diagnosis of
10 endometrial precancer and endometrial cancer and for the screening of the effects of candidate pharmacological agents on the onset, progress, and regression of endometrial precancer or endometrial cancer.

The invention also includes kits comprising the PTEN binding agents described herein. The kit includes a package housing a container that contains an agent for determining the level of PTEN in a sample. The kit may also include a control. The kit may also include instructions
15 as described herein. The instructions typically will be in written form and will provide guidance for carrying out the assay embodied by the kit and for making a determination based upon that assay.

An example of a kit may include an antibody or antigen-binding fragment thereof that binds specifically to a PTEN polypeptide, attached to a substrate (e.g. a dipstick). The
20 substrate is then applied to a sample from a patient or to a surface that may contain PTEN and the surface of the substrate is then processed to assess whether specific binding occurs between the antibody and a polypeptide or other component of the sample. As will be understood by one of skill in the art, such binding assay may also be performed with a sample or object contacted with an antibody or antigen-binding fragment thereof and/or PTEN that is
25 in solution, for example in a 96-well plate or applied directly to an object surface.

Examples

Example 1

Introduction

30 To determine the earliest stage of endometrial neoplasia in which PTEN mutation occurs, 30 hysterectomy specimens containing endometrioid endometrial adenocarcinomas and coexisting computerized morphometry-diagnosed benign or premalignant endometrial tissue were examined for the presence of mutations.

Methods

Tissue Samples

A series of paraffin embedded endometrial tissues was assembled from the pathology files of Brigham and Women's Hospital, following approval by the Human Studies Committee at that institution. The set, which consisted of tissues from 30 hysterectomies containing endometrioid endometrial adenocarcinoma, and premalignant lesions ("precancers") defined objectively by computerized morphometric analysis (12;23), was used for PTEN mutational analysis. A single region representative of each tissue diagnosis was randomly selected in each hysterectomy. One third of selected hysterectomies also contained histologically "normal" endometrium suitable for analysis, and all contained normal myometrial tissue for use as a DNA control. Endometrial polyps were excluded from analysis.

Histologic classification using computerized morphometric analysis

Diagnosis was accomplished using a combination of pathologist review, and objective computerized morphometry. First, carcinomas were distinguished from premalignant lesions by presence of at least one of three diagnostic features: myometrial invasion, solid areas of neoplastic epithelium, or extensively meandering interconnected glandular structures. Endometrial tissues judged not to constitute carcinoma were marked by circumscribing with ink on the glass slide. Computerized morphometric analysis of corresponding delineated regions on H&E stained sections was performed with the QProdit 6.1 system (Leica, Cambridge, UK) as previously (12;23;25) described. For each lesion the D-score was calculated, incorporating volume percentage stroma (VPS), standard deviation of shortest nuclear axis (SDSNA), and gland outer surface density (OUTSD), and then classed as precancers ($D < 0$), indeterminate ($0 < D < 1$), or benign ($D > 1$) based on the previously developed outcome-predictive formula $D = 0.6229 + (0.0439 \times VPS) - (3.9934 \times \ln(\text{SDSNA})) - (0.1592 \times \text{OUTSD})$ (12;23). Endometrial areas scored as benign were subclassified by pathologist review. Atrophic, cycling, or reactive endometrium was identified and grouped as "normal." Unopposed estrogen exposed endometria were diagnosed by appearance of occasional glandular cysts in a disordered proliferative field, without sufficient glandular crowding or atypia to qualify as a precancer. The source of unopposed estrogen was either endogenous (anovulatory cycles) or exogenous (pharmacological estrogens). Subjective criteria for EIN diagnosis include three steps: 1)

exclusion of overlapping benign changes such as polyps, reactive change, and confounding effects of progestin administration; 2) gland crowding, estimated by reductions in volume percentage stroma to less than half the sample volume; and 3) altered cytology.

5 *DNA isolation and PTEN mutational analysis*

Details of microsatellite instability testing (15), and clonal analysis (14;26-28) (24) of 30 hysterectomies used for PTEN DNA mutational analysis have been previously reported. DNA from desired areas of paraffin sections was isolated by selective ultraviolet irradiation (14), typically encompassing a 3mm diameter region of tissue containing dozens of individual glands. PTEN coding sequence was amplified with intron-based PCR primers thereby avoiding co-amplification of the intronless PTEN pseudogene on chromosome 9 (29). A GC-clamp at the 5' end of one PCR primer stabilized the termini of double-stranded PCR products, increasing sensitivity of mutation detection by altered migration in a chemical denaturing gradient (Denaturing Gradient Gel Electrophoresis, or "DGGE"). DGGE was performed using published methods (30). The oligonucleotide primers used are listed beginning from the 5' end: primer 1FGC, CGT CTG CCA TCT CTC TCC TCC T (SEQ ID NO: 1); primer 1RGC, CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAA ATA ATA AAT CCG TCT ACT CCC ACG TTC T (SEQ ID NO: 2); primer 2FGC, CGT CCC GCG TTT GAT TGC TGC ATA TTT CAG (SEQ ID NO: 3); primer 2RGC, CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTC TAA ATG AAA ACA CAA CAT G (SEQ ID NO: 4); primer 3FGC, CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GTA AAT GGT ATT TGA GAT TAG (SEQ ID NO: 5); primer 3RGC, GCG CGA AGA TAT TTG CAA GCA TAC A (SEQ ID NO: 6); primer 4FGC, CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAA ATA ATA AAC ATT ATA AAG ATT CAG GCA ATG (SEQ ID NO: 7); primer 4RGC, GAC AGT AAG ATA CAG TCT ATC (SEQ ID NO: 8); primer 5.1FGC, CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GTT TTT TCT TAT TCT GAG GTT ATC (SEQ ID NO: 9); primer 5.1RGC, TCA TTA CAC CAG TTC GTC C (SEQ ID NO: 10); primer 5.2FGC, TCA TGT TGC AGC AAT TCA C (SEQ ID NO: 11); primer 5.2RGC, CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GGAA GAG GAA AGG AAA AAC ATC (SEQ ID NO: 12); primer 6FGC, GCG CGT TTC AAT TTG GCT TCT CTT T (SEQ ID NO: 13); primer 6RGC, CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GAA ATA ATA AAT AAG AAA ACT GTT CCA

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ATA C (SEQ ID NO: 14); primer 7FGC, CGT CCC GCA ATA CTG GTA TGT ATT TAA
 C (SEQ ID NO: 15); primer 7RGC, CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG
 CCC CCG CCC GGA TAT TTC TCC CAA TGA AAG (SEQ ID NO: 16); primer 8FGC,
 CGG TTT CAC TTT TGG GTA AAT A (SEQ ID NO 17); primer 8RGC, CGC CCG CCG
 5 CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAC CCC CAC AAA ATG TTT
 AAT (SEQ ID NO: 18); primer 9FGC, CGC CCG CCG CGC CCC GCG CCC GGC CCG
 CCG CCC CCG CCC GTC ACT AAA TAG TTT AAG ATG (SEQ ID NO: 19); primer
 9RGC, TTC ATT CTC TGG ATC AGA GT (SEQ ID NO: 20).

PCR products were electrophoresed 16hr at 100 volts at 60°C on a 10%
 10 polyacrylamide gel containing 5% glycerol and a linear 15%-50% urea-formamide gradient.
 Samples were visualized by ultraviolet transillumination of the ethidium bromide stained gel,
 and usually appeared as “doublets” of mutant PTEN products offset by wild-type DNA
 contributed by contaminating normal tissues or the companion allele. Aberrant bands were
 reamplified and a nested sequencing primer employed for generation of fluorescent labeled
 15 terminated sequencing products that were analyzed on an automated DNA sequencer as
 previously described (6). DGGE gels and sequencing chromatograms were independently
 read by three sets of individuals.

Statistical Analysis

20 Fishers exact tests were performed on the data in Table I using SYSTAT v.9.0 (SPSS
 Inc., Chicago, IL).

Results

Somatic (occurring in tumor only) PTEN mutations were found in 25 of 30 (85%)
 25 endometrial carcinomas and 16 of 29 (55%) precancers (Table I). Two-tail Fishers exact test
 of diagnosis (cancer/EIN) by PTEN mutation (present/absent) showed that cancers had a
 significantly ($p=0.025$) increased PTEN mutation rate compared to their precursors. No
 normal endometria showed mutations in PTEN (Table I). Interestingly, among both cancers
 and precancers the majority (73% and 52%, respectively) harbored a single mutation, but two
 30 or more intragenic mutations affecting two or more exons were also observed (Table I).
 Three changes deep within introns (non-coding) were also identified. Computerized
 morphometric analysis cleanly segregates all non-cancerous PTEN mutant tissues into a
 precancer, or EIN, group (Figure 1) having a calculated D-Score <0. Morphometrically

defined precancers are usually diagnosed by practicing pathologists as atypical endometrial hyperplasias.

5 **Table 1. PTEN Mutation Endometrial Tissue Diagnosis**

Endometrial Tissue	PTEN Mutation ¹		
	n	Total with Mutations	Mutation 2 exons
Cancer, Endometrioid	30	83% (25)	10% (3)
Precancer ² (EIN)	29	55% (16)	3% (1)
Indeterminate ³	--	--	--
Unopposed Estrogen Effect ⁴	--	--	--
"Normal" ⁵	10	0%	0%
Cancer, non-Endometrioid ⁶	--	--	--

1) 30 hysterectomies with endometrioid endometrial adenocarcinoma and co-existing regions of either premalignant or benign endometrial tissue were tested for all nine PTEN exon
 10 mutations. Although the distribution of multiple exonic hits between one or two alleles is unknown it provides some indication of that fraction of cases which are candidates for biallelic mutational inactivation.

2) Precancers diagnosed by computerized morphometric analysis, D-Score <0. All cases independently confirmed by pathologist as EIN.

15 3) Indeterminate by computerized morphometry, 0<D<1. Diagnosed by pathologist as EIN (6/9), unopposed estrogen (1/9), secretory endometrium (1/9), and unknown (1/9).

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4) Benign endometria by morphometry ($D > 1$), with stigmata of unopposed estrogen.

Unstained glands were always admixed with staining glands.

5) "Normal" tissues all were benign by morphometry, and included atrophic, inactive, or cycling endometrium. One severely atrophic endometrium contained no discernable PTEN protein.

6) Non-endometrioid cancers included 2 undifferentiated carcinomas, 4 papillary serous carcinomas, and 2 malignant mixed Müllerian tumors.

Example 2

10 Introduction

PCR-based analysis to determine loss of heterozygosity of markers within or flanking PTEN was performed on the series of 30 endometrial carcinoma samples shown in Table 1.

Methods

15 For details on *Tissue Samples*, see Example 1, Methods.

Loss of Heterozygosity (LOH) Analysis

DNA from carcinoma and adjacent normal myometrium was amplified with PTEN linked primers D10S541, and D10S215 (MapPairs, Research Genetics Inc., Huntsville, AL) in the presence of ^{32}P -TTP. PCR products of these polymorphic microsatellites were resolved on non-denaturing polyacrylamide gels(31;32), and resulting autoradiograms were visually assessed using a reference set of calibrated autoradiograms(26). Tumor allele intensities were compared to those of matched normal reference myometrium and scored as "LOH positive" when there was at least a 50% reduction of one allele.

25 Results

Overall, the loss of heterozygosity frequency was 23% (7/30) and all of these samples had PTEN mutations in the remaining allele indicating inactivation of both PTEN alleles. PCR testing for loss of heterozygosity of markers within or flanking PTEN showed an overall 10q23 LOH rate of 23% (7/30). One third (7/22) of cancers with intragenic mutations within a single PTEN exon had loss of the second allele by deletion.

Example 3

Introduction

The possibility of biallelic inactivation of PTEN and resultant lack of PTEN protein expression was assessed through the use of immunocytochemistry.

Methods

5 *Tissue Samples*

A second series of paraffin embedded endometrial tissues was assembled from the pathology files of Brigham and Women's Hospital, following approval by the Human Studies Committee at that institution. This series of endometrial tissues from 58 patients (38 hysterectomies, 20 curettings/biopsies) was used for PTEN immunohistochemistry. Cases
10 were selected by report review for diagnoses of endometrial adenocarcinoma and/or anovulatory/hyperplastic endometrium.

Immunohistochemistry

Monoclonal antibody 6H2.1 raised against the last 100 C-terminal amino acids of
15 PTEN (33) was used in all immunocytochemical analyses. Specificity has previously been demonstrated by Western blot of wild type and PTEN null cell lines, and successful blockade of paraffin section immunohistochemistry signal upon antibody pre-incubation with competing synthetic PTEN peptide (33).

Formalin fixed tissue samples were embedded in paraffin following standard
20 histologic practices. Immunostaining was performed using a microwave antigen-retrieval protocol as described (33). Sections were incubated with monoclonal antibody 6H2.1 (dilution 1:100) for 1 hour at room temperature, washed, incubated with a secondary biotinylated horse anti-mouse IgG. Signal was detected by addition of avidin peroxidase and a chromogenic reaction carried out with 3-3' diaminobenzidine, which gives a brown reaction
25 product. Intensity of epithelial staining was scored in methyl green counterstained slides from 0 (absent) to 3 (intense). PTEN immunohistochemistry using the 6H2.1 antibody requires freshly cut paraffin sections from recently embedded (within 6-12 months) tissues in order to maximize signal. Endometrial stroma and/or normal endometrial epithelium provided an internal positive control, and negative controls without addition of primary antibody showed
30 low background in all cases.

Results

Table 1 and Figures 2 and 3 illustrate the salient PTEN immunohistochemical findings in malignant, premalignant, and estrogen driven endometria which were consistently seen in

multiple independent patients with similar lesions. Endometrioid endometrial adenocarcinomas lost PTEN protein more frequently (61%) than non-endometrioid carcinomas (25%), although the small series does not show statistical significance (two-tailed Fishers exact test of tumor type by PTEN expression, $p=0.115$). Adjacent endometrial stroma was moderately PTEN positive, as were endothelial cells of blood vessels in immediate proximity to the tumor (Figure 2, Panel A). Precancerous (EIN) lesions had no discernible PTEN protein in 75% of cases, most commonly in closely packed expanses of PTEN unstained glands offset by dispersed benign glands having a different cytology (Figure 2, panels B-C). A less frequent pattern of heterogenous PTEN staining was seen in some "transitional" benign-precancer examples without cytologic changes (Figure 3, Panels A-C). Two-tail Fishers exact test of cancer/EIN diagnosis by PTEN protein expression showed a non-significant ($p=0.491$) difference, although the small sample size limits the power of this comparison.

Whereas some unopposed estrogen-exposed endometria maintained ubiquitous epithelial PTEN expression, 28% had a background of PTEN staining glands punctuated by scattered negative glands. Figure 3 shows endometria with heterogenous PTEN protein expression in which PTEN-negative glands may (Figure 3, Panel E-F) or may not (Figure 3, Panel B-C) display a cytology different than the surrounding PTEN expressing epithelia. Most areas of tubal change in disordered proliferative endometrium continue to express PTEN protein.

Table 2. PTEN Expression by Endometrial Tissue Diagnosis

Endometrial Tissue	n	Absent	Mild	Moderate	Intense
Cancer, Endometrioid	33	61% (20)	12% (4)	24% (8)	3% (1)
Precancer ² (EIN)	12	75% (9)	8% (1)	17% (2)	0%
Indeterminate ³	9	56% (5)	11% (1)	33% (3)	0%
Unopposed Estrogen Effect ⁴	7	28.5% (2)	0%	28.5% (2)	43% (3)
"Normal" ⁵	20	5% (1)	45% (9)	45% (9)	5% (1)

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Cancer, non-Endometrioid ⁶	8	25% (2)	25% (2)	25% (2)	25% (2)
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1) Antibody 6H2.1 immunohistochemistry, scored in epithelial cells.

2) Precancers diagnosed by computerized morphometric analysis, D-Score <0. All cases independently confirmed by pathologist as EIN.

5 3) Indeterminate by computerized morphometry, $0 < D < 1$. Diagnosed by pathologist as EIN (6/9), unopposed estrogen (1/9), secretory endometrium (1/9), and unknown (1/9).

4) Benign endometria by morphometry ($D > 1$), with stigmata of unopposed estrogen.

Unstained glands were always admixed with staining glands.

5) "Normal" tissues all were benign by morphometry, and included atrophic, inactive, or
10 cycling endometrium. One severely atrophic endometrium contained no discernable PTEN protein.

6) Non-endometrioid cancers included 2 undifferentiated carcinomas, 4 papillary serous carcinomas, and 2 malignant mixed Müllerian tumors.

15 **Example 4**

Introduction

Case Selection

132 paraffin-embedded endometrial biopsies and curettings obtained in the years 1998-2000 (Department of Pathology, Brigham and Women's Hospital, Boston, MA) were
20 allocated to proliferative, persistent proliferative, or EIN diagnostic classes based upon slide review consensus of two gynecologic pathologists. "Normal" proliferative endometria all came from premenopausal women less than 40 years of age (average age of 34.0 ± 4.5) who were not taking supplemental hormones. Persistent proliferative endometria (mean age 45.2 ± 9.3) had mitotically active but cytologically uniform glands with occasional cystically
25 dilated glands, and were ascribed either to endogenous (anovulation) or exogenous (pharmacologic) estrogen sources based on clinical history. Endometrial polyps disqualified a case from the proliferative and persistent proliferative categories. EIN diagnosis (mean age 54.1 ± 7.8) was made visually according to published criteria (41). The WHO endometrial hyperplasia classification system was not used in these studies because of its poor
30 reproducibility, and discordance with discrete biologic groups defined by genetic analysis

(40).

Ninety repeat biopsies were retrieved by diagnostic review from 45 individual women with proliferative endometrium on more than one occasion. Most repeat biopsies were symptomatically indicated (usually bleeding), but some were incidental to unrelated findings such as uterine fibroids, polyps.

Immunohistochemistry

Dewaxed rehydrated 4 µm paraffin sections underwent microwave antigen retrieval before adding primary anti-PTEN antibody 6H2.1 (Cascade Biosciences, Winchester, MA, Cat.#ABM-2052) at 1:300 dilution. Anti-estrogen receptor antibody ER-ID5 (Dako), and anti-progesterone receptor antibody IA6 (Dako) were used at 1:300 and 1:100 dilutions, respectively. Primary antibody was incubated overnight at 4°C, washed, incubated with appropriate secondary biotinylated immunoglobulin (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA) and signal was detected by sequential addition of avidin peroxidase and 3,3'-diaminobenzidine. Epithelial staining was scored by two pathologists using endometrial stroma and/or normal endometrial epithelium as an internal positive control and negative run controls without addition of primary antibody. All tissue fragments were examined, and individual glands were scored as PTEN null when signal was absent in the nuclear and cytoplasmic compartments of most cells in that gland. Hormone receptors were scored by signal intensity in the nuclear compartment.

Genomic Analysis

Matched PTEN expressing and non-expressing proliferating endometrial epithelial cells were sampled using laser capture microdissection directed by PTEN immunohistochemistry of flanking serial sections. Approximately 10-50ng of DNA per sample was PCR amplified using primers which define the coding region and flanking introns of all 9 *PTEN* gene exons. PCR products were subjected to denaturing gradient gel electrophoresis (DGGE), which was virtually 100% sensitive and specific in detecting sequence-confirmed PTEN mutations (37). DNA samples showing DGGE variants are re-subjected to PCR and semi-automated direct sequencing (ABI377a or PE3600 sequencers, Applied Biosystems, Foster City, CA).

For each patient, DNA from PTEN expressing and non-expressing epithelial cells was subjected to PTEN deletional analysis by PCR using 5*-tagged fluorophor primers, which

amplify microsatellites flanking and within the *PTEN* gene, D10S579, D10S2491, and D10S541, and then electrophoresed through an ABI377a gel and analyzed with GeneScan software (42). Marker heterozygosity manifested as two peaks on a GeneScan gel, representing two different alleles present at that marker. Matched sets of DNA samples from PTEN expressing and non-expressing glands were compared at each marker and if one peak was reduced by at least a third, loss of heterozygosity (LOH) had occurred, which represented deletion of one of the alleles and usually, that chromosomal region.

Morphometry

A 1-mm circular window (surface area 0.785mm^2) containing 100 randomly distributed points was superimposed on digitized photomicrographs of PTEN immunohistochemically stained endometria, and points over the fragment of interest (PTS100) tallied by composition of underlying tissue (stroma, STROMA 100; PTEN-expressing or "Positive" glands, POS100; PTEN-null glands, NULL100). Excluded were 7 fragmented or small ($< 1/2$ sample window) samples, 4 cases diagnosed on hematoxylin and eosin slides as EIN in which the targeted PTEN-null glands did not involve the EIN focus, and one PTEN-null EIN focus which was distorted by tangential sectioning on recut. Surface area assigned to glands included combined epithelial and luminal compartments. Geometric centroids of each gland profile were marked, and the number of PTEN null (NULLCT) and expressing (POSCT) gland centroids within the window counted. Variables were calculated as follows: 1) VPS= $100 \times (\text{STROMA100}/\text{PTS100})$; 2) VPNULL= $100 \times (\text{NULL100}/\text{PTS100})$; 3) VPPOS= $100 \times (\text{POS100}/\text{PTS100})$; 4) DENNULL= $(\text{NULLCT}/\text{PTS100}) \times (100 \text{ points in window}/0.785\text{mm}^2 \text{ window size})$; 5) DENPOS= $(\text{POSCT}/\text{PTS100}) \times (100 \text{ points in window}/0.785\text{mm}^2 \text{ window size})$; 6) SZNULL= $(\text{NULL100}/\text{NULLCT})(0.785\text{mm}^2/100 \text{ points in window})$; 7) SZPOS= $(\text{POS100}/\text{NULLCT})(0.785\text{mm}^2/100 \text{ points in window})$.

RESULTS

PTEN-null endometrial rates were 43, 56, and 63% in proliferative, persistent proliferative, and EIN diagnostic categories respectively (Table 3, Figure 4). There was a linear trend by decade of age for increasing PTEN-null rates in older women (Coachman's test of linear trend, $p=0.014$). Average age of women with and without PTEN-null glands was 43.8 (± 9.7) and 40.2 (± 11.6) years, respectively. PTEN-null glands in the three diagnostic groups are present in women biopsied for a variety of reasons, so these results are applicable

to a broad range of women seeking routine medical care (Table 3).

Table 3: Clinicopathologic features of endometria by PTEN immunohistochemistry, and slide diagnosis

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		% PTEN-null by Diagnosis (fraction)			Total
		Proliferative	Persistent Proliferative	EIN	
Sample	Curettage	42.9 (6/14)	54.5 (6/11)	75.0 (6/8)	54.5 (18/33)
	Biopsy	42.9 (18/42)	56.7(17/30)	59.3 (16/27)	51.5 (51/99)
Clinical Indication	Bleeding	42.9 (12/28)	56.3 (18/32)	55.0 (11/20)	51.3 (41/80)
	Infertility/ recurrent abortion	41.7 (5/12)	0.0 (0/0)	100.0 (1/1)	46.2 (6/13)
	Prior hyperplasia	0.0 (0/1)	0.0 (0/0)	71.4 (5/7)	75.0 (6/8)
	Anatomic (fibroids, septum, polyp, thick stripe)	50.0 (3/6)	100.0 (1/1)	100.0 (1/1)	62.5 (5/8)
	Other (pain, endometriosis)	20 (1/5)	33.3 (1/3)	0.0 (0/2)	20.0 (2/10)
	Unspecified	75.0 (3/4)	60.0 (3/5)	100 (4/4)	76.9 (10/13)
Hormones	Endogenous	42.9 (24/56)	55.9 (19/34)	64.3 (18/28)	51.7 (61/118)
	Exogenous	0.0 (0/0)	57.1 (4/7)	57.1 (4/7)	57.1 (8/14)
Menopause	Pre	42.9 (24/56)	57.1 (20/35)	66.7 (14/21)	51.8 (58/112)
	Post	0.00 (0/0)	50.0 (3/6)	57.1 (8/14)	55.0 (11/20)
Total		42.9 (24/56)	56.1 (23/41)	62.9 (22/35)	52.3 (69/132)

The occurrence of PTEN-null glands in 43% (24/56) of histologically normal proliferative endometrium (confirmed by staining two sections in each case) was unexpectedly high. In general, only a few histologically unaltered glands were PTEN-null among hundreds of proliferating glands in these otherwise unremarkable endometria. Since

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PTEN expression responds to the hormonal environment (43), estrogen and progesterone receptor immunohistochemistry were performed on flanking serial tissue sections and showed in all cases that the PTEN null and expressing glands in proliferative endometria retained comparable receptor quantities. Nineteen out of 24 proliferative endometria with PTEN-null
5 glands had sufficient material for microdissection. Matched DNA from PTEN expressing and nonexpressing glands from the same patient were co-processed for direct comparison of PTEN mutation and deletion (Tables 4 and 5). All PTEN-expressing matched control glands had a wild-type (normal) genotype whereas 84% (16/19) of non-expressing glands had a mutation (n=8) and/or loss of at least one 10q23 heterozygous marker (n = 13) in the region
10 of the *PTEN* locus. Results of denaturing gradient gel electrophoresis (DGGE), on products from single plex PCR on the exon 5 of the PTEN gene are shown in Figure 6.

Table 4: PTEN Mutations in Proliferative Endometria: *PTEN* mutation, deletion and expression status.

Patient #	Expression*	Mutation	LOH Status at Markers**:		
			D10S579	D10S2491	D10S541
704	+	None	NI	NI	ROH
704	-	None	NI	NI	LOH
339	+	None	ROH	ROH	NI
339	-	Y88S	LOH	ROH	NI
521	+	None	ROH	NI	ROH
521	-	None	LOH	NI	LOH
366	+	None	NI	NI	ROH
366	-	IVS4-1G>T	NI	NI	LOH
932	+	None	NI	ROH	NI
932	-	c963-8InsA	NI	ROH	NI
603	+	None	ROH	ROH	ROH
603	-	c462-473del12	LOH	LOH	LOH
471	+	None	ROH	ROH	ROH
471	-	C643-5delT	LOH	ROH	ROH
253	+	None	ROH	NI	NI
253	-	None	LOH	NI	NI
479	+	None	NI	ROH	ROH
479	-	None	NI	ROH	LOH
071	+	None	NI	ROH	NI
071	-	None	NI	ROH	NI
075	+	None	ROH	NI	NI
075	-	c462-473del12	LOH	NI	NI
908	+	None	ROH	ROH	ROH
908	-	None	LOH	LOH	LOH
106	+	None	ROH	ROH	NI
106	-	None	LOH	LOH	NI
613	+	None	NI	ROH	ROH
613	-	None	NI	ROH	ROH
039	+	None	NI	ROH	ROH
039	-	None	NI	ROH	LOH
229	+	None	ROH	ROH	ROH
229	-	C988-90InsA	ROH	ROH	ROH
421	+	None	ROH	ROH	ROH
421	-	IVS4-1G>A	ROH	ROH	ROH
717	+	None	ROH	ROH	ROH
717	-	None	ROH	ROH	LOH
469	+	None	ROH	ROH	ROH
469	-	None	ROH	ROH	ROH

* PTEN protein Null (-) and Positive (+) glands from individual biopsies.

5 ** LOH, Loss of Heterozygosity (NI=not informative, ROH=retention of heterozygosity, LOH=loss of heterozygosity).

**Table 5: Morphometric Characteristics of PTEN-null Glands,
by Diagnosis within the Morphometry Window**

PTEN Parameter	Abbrev.	Units	Proliferative mean (SD)	Persistent Proliferative Mean (SD)	EIN mean (SD)	p*
Sample	n	Patients	20	20	17	
Volume % Stroma	VPS	percentage	78.3 (10.2)	65.2 (13.7)	35.0 (7.9)	<0.001
Volume % Null Gland	VPNULL	percentage	13.0 (10.1)	25.6 (17.7)	63.9 (8.7)	<0.001
Volume % Positive Glands	VPPOS	percentage	8.7 (5.4)	9.2 (1.1)	1.1 (1.9)	0.001
Density of Null Glands	DENNULL	# glands/mm ²	8.4 (5.1)	9.8 (6.6)	26.2 (8.0)	<0.001
Density of Positive Glands	DENPOS	# glands/mm ²	10.5 (8.7)	3.5 (3.3)	0.9 (1.7)	<0.001
Size of Null Glands	SZNULL	mm ² per gland	0.016 (0.010)	0.030 (0.017)	0.026 (0.007)	<0.004
Size of Positive Glands	SZPOS	mm ² per gland	0.014 (0.015)	0.024 (0.012)	0.018 (0.015)	<0.090

5 *two-tailed ANOVA, significance

The appearance of rare histologically normal glands harboring *PTEN* mutations would be inconsequential if they are completely shed with normal menstruation. Thus, *PTEN* immunohistochemistry was performed on 34 premenopausal women (no hormonal therapy, average age 42.3 ± 6.2 years) with unremarkable proliferative endometrium on two separate occasions (interval averaged 400 days, range 26-1167 days). Twelve of 34 women had *PTEN*-null glands initially (Table 6), scattered throughout varying depths of the endometrial thickness, and 83% (10/12) of these continued to be present on follow-up. *PTEN* status of paired biopsies in Table 6 is highly associated with initial phenotype (Fishers exact test p=0.01, with an Odds Ratio of 10.71). A woman with *PTEN*-null glands in her endometrium is five times more likely to have *PTEN*-null glands on repeat biopsy than not. A separate series of 11 postmenopausal women (mean age 58.1 ± 2.6 years) with two proliferative endometria separated by an average of 494 days (range 142-985 days) and sampled during the

estrogenic phase of sequential estrogen/progestin replacement therapy were used for PTEN immunohistochemistry. Two had PTEN-null glands initially, retained by one on follow-up. 11% (1/9) with PTEN expressing first biopsies developed PTEN-null glands in the second biopsy. These 11 patients are not shown in Table 6, but when pooled with those of Table 6 increased the association of PTEN status between first and second biopsy (Fishers exact $p < 0.001$, Odds Ratio 17.3).

Table 6: PTEN status in repeat biopsies of premenopausal women with endogenously cycling proliferative endometrium*.

	2 nd Sample PTEN-positive	2 nd Sample PTEN-null	Total
1 st Sample PTEN-positive	15	7	22
1 st Sample PTEN-null	2	10	12
Total	17	17	34

*Initial (1st sample) and repeat (2nd sample) endometrial samples scored as PTEN-nonexpressing (null) or having only PTEN-expressing glands (positive).

Changes in the histological structure of PTEN-null clones were documented by morphometric analysis of PTEN-immunostained normal proliferative, persistent proliferative, and EIN endometria (Figure 5).

REFERENCES

- (1) Risinger JI, Hayes AK, Berchuck A, Barrett JC. PTEN/MMAC1 mutations in endometrial cancers. Cancer Res 1997; 57:4736-4738.
- (2) Tashiro H, Blazes MS, Wu R, Cho KR, Bose S, Wang SI et al. Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies. Cancer Res 1997; 57:3935-3940.
- (3) Peiffer SL, Herzog TJ, Tribune DJ, Mutch DG, Gersell DJ, Goodfellow PJ. Allelic loss of sequences from the long arm of chromosome 10 and replication errors in endometrial cancers. Cancer Res 1995; 55:1922-1926.
- (4) Nagase S, Yamakawa H, Sato S, Yajima A, Horii A. Identification of a 790-kilobase region of common allelic loss in chromosome 10q25-q26 in human endometrial cancer. Cancer Res 1997; 57:1630-1633.

- (5) Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM et al. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci U S A 1999; 96:1563-8.
- 5 (6) Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nature Genet 1997; 16:64-67.
- (7) Marsh D, Coulon V, Lunetta K, Rocca-Serra P, Dahia P, Zheng Z et al. Mutation spectrum and genotype-phenotype analyses in Cowden Disease and Bannayan-Zonana Syndrome, 2 hamartoma syndromes with germline PTEN mutation. Hum Mol Genet 10 1998; 7:507-515.
- (8) Eng C. Genetics of Cowden syndrome: through the looking glass of oncology. Int J Oncol 1998; 12:701-710.
- (9) Silverberg S, Sasano N, Yajima A. Endometrial carcinoma in Miyagi Prefecture, Japan: histopathologic analysis of a cancer-based series and comparison with cases in 15 American women. Cancer (Phila) 1982; 49:1504-1510.
- (10) Sherman ME, Sturgeon S, Brinton L, Kurman RJ. Endometrial cancer chemoprevention: Implications of diverse pathways of carcinogenesis. J Cell Biochem 1995; 59 Suppl. 23:160-164.
- (11) Parazzini F, La Vecchia C, Bocciolone L, Franceschi S. The epidemiology of 20 endometrial cancer. Gynecol Oncol 1991; 41:1-16.
- (12) Baak JPA, Nauta J, Wisse-Brekelmans E, Bezemer P. Architectural and nuclear morphometrical features together are more important prognosticators in endometrial hyperplasias than nuclear morphometrical features alone. J Pathol 1988; 154:335-341.
- (13) Mutter GL, Wada H, Faquin W, Enomoto T. K-ras mutations appear in the 25 premalignant phase of both microsatellite stable and unstable endometrial carcinogenesis. Mol Pathol 1999; 52:257-262.
- (14) Jovanovic AS, Boynton KA, Mutter GL. Uteri of women with endometrial carcinoma

contain a histopathologic spectrum of monoclonal putative precancers, some with microsatellite instability. *Cancer Res* 1996; 56:1917-1921.

- 5 (15) Mutter GL, Boynton KA, Faquin WC, Ruiz RE, Jovanovic AS. Allelotype mapping of unstable microsatellites establishes direct lineage continuity between endometrial precancers and cancer. *Cancer Res* 1996; 56:4483-4486.
- (16) Scully RE, Bonfiglio TA, Kurman RJ, Silverberg SG, Wilkinson EJ. Uterine corpus. *Histological Typing of Female Genital Tract Tumors*. New York: Springer-Verlag, 1994: 13-31.
- 10 (17) Winkler B, Alvarez S, Richart R, Crum C. Pitfalls in the diagnosis of endometrial neoplasia. *Obstet Gynecol* 1984; 64:185-194.
- (18) Kendall BS, Ronnett BM, Isacson C, Cho KR, Hedrick L, Diener-West M et al. Reproducibility of the diagnosis of endometrial hyperplasia, atypical hyperplasia, and well-differentiated carcinoma. *Am J Surg Pathol* 1998; 22:1012-1019.
- 15 (19) Maxwell G, Risinger J, Gumbs C, Shaw H, Bentley R, Barrett J et al. Mutation of the PTEN tumor suppressor gene in endometrial hyperplasias. *Cancer Res* 1998; 58:2500-2503.
- (20) Levine RL, Cargile CB, Blazes MS, Van Rees B, Kurman RJ, Ellenson LH. PTEN mutations and microsatellite instability in complex atypical hyperplasia, a precursor lesion to uterine endometrioid carcinoma. *Cancer Res* 1998; 58:3254-3258.
- 20 (21) Yoshinaga K, Sasano H, Furukawa T, Yamakawa H, Yuki M, Sato S et al. The PTEN, BAX, and IGFIIR genes are mutated in endometrial atypical hyperplasia. *Jpn J Cancer Res* 1998; 89(10):985-990.
- 25 (22) Colgan TJ, Norris HJ, Foster W, Kurman RJ, Fox CH. Predicting the outcome of endometrial hyperplasia by quantitative analysis of nuclear features using a linear discriminant function. *Int J Gynecol Pathol* 1983; 1:347-352.
- (23) Dunton C, Baak J, Palazzo J, van Diest P, McHugh M, Widra E. Use of computerized morphometric analyses of endometrial hyperplasias in the prediction of coexistent

- cancer. *Am J Obstet Gynecol* 1996; 174:1518-1521.
- (24) Mutter GL, Baak JPA, Crum CP, Richart RM, Ferenczy A, Faquin WC. Endometrial precancer diagnosis by histopathology, clonal analysis, and computerized morphometry. *J Pathol* 2000; (In Press).
- 5 (25) Baak JPA. *Manual of Quantitative Pathology in Cancer Diagnosis and Prognosis*. 1991. New York, Springer-Verlag.
- (26) Mutter GL, Boynton KA. X chromosome inactivation in the normal female genital tract: Implications for identification of neoplasia. *Cancer Res* 1995; 55:5080-5084.
- (27) Mutter GL, Chaponot M, Fletcher J. A PCR assay for non-random X chromosome
10 inactivation identifies monoclonal endometrial cancers and precancers. *Am J Pathol* 1995; 146:501-508.
- (28) Mutter GL, Boynton KA. PCR bias in amplification of androgen receptor alleles, a trinucleotide repeat marker used in clonality studies. *Nucleic Acids Res* 1995; 23:1411-1418.
- 15 (29) Dahia P, Fitzgerald M, Zhang X, Marsh D, Zheng Z, Pietsch T et al. A highly conserved processed PTEN pseudogene is located on chromosome band 9p21. *Oncogene* 1998; 16:2403-2406.
- (30) Marsh DJ, Dahia PL, Caron S, Kum JB, Frayling IM, Tomlinson IP et al. Germline PTEN mutations in Cowden syndrome-like families. *J Med Genet* 1998; 35:881-885.
- 20 (31) Pinto AP, Lin MC, Mutter GL, Sun D, Villa LL, Crum CP. Allelic loss in human papillomavirus-positive and -negative vulvar squamous cell carcinomas. *Am J Pathol* 1999; 154:1009-15.
- (32) Lin MC, Mutter GL, Trivijisilp P, Boynton KA, Sun D, Crum CP. Patterns of allelic loss (LOH) in vulvar squamous carcinomas and adjacent noninvasive epithelia. *Am J
25 Pathol* 1998; 152:1313-1318.
- (33) Perren A, Weng L, Boag A, Ziebold U, Thakore K, Dahia P et al. Immunocytochemical evidence of loss of PTEN expression in primary ductal

adenocarcinomas of the breast. *Am J Pathol* 1999; 155:1253-1260.

- (34) Bergeron C, Nogales F, Masseroli M, Abeler V, Duvillard P, Muller-Holzner E et al. A multicentric European study testing the reproducibility of the WHO Classification of endometrial hyperplasia with a proposal of a simplified working classification for biopsy and curettage specimens. *Am J Surg Pathol* 1999; 23:1102-1108.
- (35) Gray L, Christopherson W, Hoover R. Estrogens and endometrial carcinoma. *Obstet Gynecol* 1977; 49:385-389.
- (36) Weiss N, Sayvetz T. Incidence of endometrial cancer in relation to the use of oral contraceptives. *N Engl J Med* 1980; 302:551-554.
- (37) Dahia PLM, Aguiar RCT, Alberta J, Kum JB, Caron S, Sill H et al. PTEN is inversely correlated with the cell survival factor Akt/PKB and is inactivated via multiple mechanisms in haematological malignancies. *Hum Mol Genet* 1999; 8:185-193.
- (38) Eng C, Vigg J. Genetic testing: the problems and the promise. *Nat Biotechnol* 1997; 15:422-426.
- (39) FitzGerald MG, Marsh DJ, Wahrer D, Bell D, Caron S, Shannon KE et al. Germline mutations in PTEN are an infrequent cause of genetic predisposition to breast cancer. *Oncogene* 1998; 17:727-731.
- (40) Mutter, G. L., Baak, J. P. A., Crum, C. P., Richart, R. M., Ferenczy, A., and Faquin, W. C. Endometrial precancer diagnosis by histopathology, clonal analysis, and computerized morphometry. *J. Pathol.*, 190: 462-469, 2000.
- (41) Mutter, G. L. Histopathology of genetically defined endometrial precancers. *Int. J. Gynecological Pathology*, 19: 301-309, 2000.
- (42) Marsh, D., Dahia, P., Coulon, V., Zhong, Z., Dorion-Bonnett, F., Call, K., Little, R., Lin, A., Goldstein, A., Eeles, R., Hodgson, S., Richardson, A., Robinson, B., Weber, H., Longy, M., and Eng, C. Allelic imbalance, including deletion of PTEN/MMAC I at the Cowden disease locus on 10q22-23 in hamartomas from patients with Cowden disease

-43-

and germline PTEN mutation. Genes Chrom.Cancer, 21: 61-69, 1998.

(43) Mutter, G. L., Lin, M. C., Fitzgerald, J. T., Kum, J. B., Ziebold, U., and Eng, C. Changes
in endometrial PTEN expression throughout the human menstrual cycle. J.

5 Clin.Endocrinol.Metab., 85: 2334-2338, 2000.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than
routine experimentation, many equivalents to the specific embodiments of the invention
10 described herein. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference in their entirety.

We claim:

Claims

1. A method for determining the likelihood of a group of endometrial cells or an endometrial gland to become cancerous, comprising
performing immunohistochemistry on a group of endometrial cells or one or more
5 endometrial glands using PTEN antibody or an antigen-binding fragment thereof and
determining the binding of the PTEN antibody or antigen-binding fragment thereof to
the group of endometrial cells or glands, wherein a reduced amount of PTEN antibody or
antigen-binding fragment thereof bound to the group of endometrial cells or glands relative to
a control group of cells indicates that the group of endometrial cells or glands has an
10 increased likelihood of becoming cancerous.
2. The method of claim 1, wherein the antibody or antigen-binding fragment thereof is
6H2.1 antibody or an antigen-binding fragment thereof.
- 15 3. The method of claim 1, wherein the group of endometrial cells or the one or more
endometrial glands and the control group of cells are present in a tissue sample.
4. The method of claim 3, wherein the tissue sample is a tissue biopsy.
- 20 5. The method of claim 1, wherein the control group of cells and the group of
endometrial cells or glands are the same cell type and wherein the amount of binding of the
PTEN antibody or antigen-binding fragment thereof to the group of endometrial cells or
glands is 50% or less of the binding of the PTEN antibody or antigen-binding fragment
thereof to the control group of cells.
- 25 6. The method of claim 1, further comprising determining the size of a group of
endometrial cells or one or more endometrial glands which have reduced PTEN expression,
wherein an increased size of the group of endometrial cells or the glands relative to a control
group of cells or glands indicates that the group of endometrial cells or glands has an
30 increased likelihood of becoming cancerous.
7. The method of any of claims 1-6, wherein the group of endometrial cells or glands are
obtained from a subject suspected of having endometrial cancer.

8. The method of any of claims 1-6, wherein the group of endometrial cells or glands are obtained from a subject having or suspected of having elevated unopposed estrogen levels.
- 5 9. The method of claim 1, wherein the subject is receiving or has received unopposed estrogen treatment.
10. A method for determining regression, progression or onset of a condition characterized by abnormal levels of PTEN protein comprising;
10 obtaining a level of the amount of PTEN from a sample obtained from a subject, and comparing the level to a control as a determination of regression, progression or onset of the condition.
11. The method of claim 10, wherein the antibody or antigen-binding fragment thereof is
15 6H2.1 antibody or an antigen-binding fragment thereof.
12. The method of claim 10, wherein the subject is undergoing drug therapy for a condition characterized by abnormal levels of PTEN protein.
- 20 13. A method for monitoring the progression of endometrial precancers, comprising, determining the expression of PTEN in endometrial cells or glands by immunohistochemistry of an endometrial tissue sample obtained at a first time using a PTEN antibody or an antigen-binding fragment thereof,
determining the expression of PTEN in endometrial cells or glands by
25 immunohistochemistry of an endometrial tissue sample obtained at a second time using the PTEN antibody or antigen-binding fragment thereof,
comparing the expression of PTEN in the endometrial cells or glands at the first time and the second time, wherein reduced expression of PTEN at the second time relative to the first time indicates progression of endometrial precancers to a cancerous stage.
- 30 14. The method of claim 13, wherein the antibody or antigen-binding fragment thereof is 6H2.1 antibody or antigen-binding fragment thereof.

15. The method of claim 13, further comprising
determining the size of groups of endometrial cells or glands which have reduced
PTEN expression in the endometrial tissue sample obtained at the first time and the
endometrial tissue sample obtained at the second time, and
5 comparing the size of the groups of endometrial cells or the glands which have
reduced PTEN expression at the first time and the second time, wherein increased size of the
groups of endometrial cells or the glands which have reduced PTEN expression at the second
time relative to the first time indicates progression of endometrial precancers to a cancerous
stage.
- 10 16. The method of any of claims 13-15, wherein the endometrial tissue samples are
obtained from a subject suspected of having endometrial cancer.
17. The method of any of claims 13-15, wherein the endometrial tissue samples are
15 obtained from a subject having or suspected of having elevated unopposed estrogen levels.
18. The method of claim 17, wherein the subject is receiving or has received unopposed
estrogen treatment.
- 20 19. The method of claim 13, wherein the subject is undergoing drug therapy for
endometrial precancer or endometrial cancer.
20. A method for monitoring the regression of endometrial precancers, comprising,
determining the expression of PTEN in endometrial cells or glands by
25 immunohistochemistry of an endometrial tissue sample obtained at a first time using a PTEN
antibody or an antigen-binding fragment thereof,
determining the expression of PTEN in endometrial cells or glands by
immunohistochemistry of an endometrial tissue sample obtained at a second time using the
PTEN antibody or antigen-binding fragment thereof,
30 comparing the expression of PTEN in the endometrial cells or glands at the first time
and the second time, wherein increased expression of PTEN at the second time relative to the
first time indicates regression of endometrial precancers from a cancerous stage.

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21. The method of claim 20, wherein the antibody or antigen-binding fragment thereof is 6H2.1 antibody or antigen-binding fragment thereof.
22. The method of claim 20, further comprising
5 determining the size of groups of endometrial cells or glands which have increased PTEN expression in the endometrial tissue sample obtained at the first time and the endometrial tissue sample obtained at the second time, and
comparing the size of the groups of endometrial cells or the glands which have
increased PTEN expression at the first time and the second time, wherein decreased size of
10 the groups of endometrial cells or the glands which have increased PTEN expression at the second time relative to the first time indicates regression of endometrial precancers from a cancerous stage.
23. The method of any of claims 20-22, wherein the endometrial tissue samples are
15 obtained from a subject suspected of having endometrial cancer.
24. The method of any of claims 20-22, wherein the endometrial tissue samples are obtained from a subject having or suspected of having elevated unopposed estrogen levels.
- 20 25. The method of claim 24, wherein the subject is receiving or has received unopposed estrogen treatment.
26. The method of claim 20, wherein the subject is undergoing drug therapy for endometrial precancer or endometrial cancer.
- 25 27. A method of selecting a treatment for endometrial precancer or endometrial cancer in a subject comprising;
obtaining a level of PTEN expression from an endometrial tissue sample obtained
from the subject by immunohistochemical analysis, and
30 selecting the treatment for endometrial precancer or endometrial cancer in the subject based at least in part on the level obtained.
28. The method of claim 27, wherein the subject is already receiving drug therapy for

endometrial precancer or endometrial cancer.

29. The method of claim 27, wherein the immunohistochemical analysis is performed using 6H2.1 clone PTEN-reactive monoclonal antibody.

5

30. A kit for identifying endometrial precancer cells in an endometrial tissue sample, comprising

a container containing a PTEN antibody or antigen-binding fragment thereof,

a second container containing a detectable compound for detecting the presence of the

10 PTEN antibody or antigen-binding fragment thereof in a sample, and

instructions for binding the PTEN antibody or antigen-binding fragment thereof to an endometrial tissue sample and for measuring the amount of PTEN antibody or antigen-binding fragment thereof bound to the endometrial tissue sample using the detectable compound.

15

31. The kit of claim 30, further comprising a container containing a second detectable compound for histological determination of the size of groups of cells or glands in the endometrial tissue sample.

20 32. A kit for identifying endometrial precancer cells in an endometrial tissue, comprising a container containing a PTEN antibody or antigen-binding fragment thereof conjugated to a detectable compound,

a second container containing a second detectable compound for histological determination of the size of groups of cells or glands in the endometrial tissue sample and

25 instructions for binding the PTEN antibody or antigen-binding fragment thereof to an endometrial tissue sample and for measuring the amount of PTEN antibody or antigen-binding fragment thereof bound to the endometrial tissue sample using the detectable compound.

30 33. A method for diagnosing endometrial precancer in a subject comprising obtaining a biological sample of endometrial tissue or cells from a subject, contacting the sample with an endometrial cell marker that specifically binds to endometrial cells,

contacting the sample with an antibody or antigen-binding fragment thereof that specifically binds PTEN,

determining specific binding between the antibody or antigen-binding fragment thereof and PTEN in the sample, and

5 determining the specific binding between the endometrial cell marker, and agents in the sample.

34. The method of claim 33, further comprising

10 comparing the determination of specific binding of the antibody or antigen-binding fragment thereof and the specific binding of the endometrial cell marker in the sample, to the specific binding of the antibody or antigen-binding fragment thereof and the specific binding of the endometrial cell marker in a control group of cells as a diagnosis for endometrial precancer in the subject.

15 35. The method of claim 33, wherein the antibody or antigen-binding fragment thereof is 6H2.1 antibody or an antigen-binding fragment thereof.

36. The method of claim 33, wherein the endometrial cell marker is selected from the group consisting of: antibodies and antigen-binding fragments thereof, and ligands.

20

37. The method of claim 33, wherein the endometrial cell marker comprises an anti-estrogen receptor antibody or an anti-progesterone receptor antibody.

38. The marker of claim 37, wherein the anti-estrogen receptor antibody is ER-ID5.

25

39. The marker of claim 37, wherein the anti-progesterone receptor antibody is IA6.

40. The method of claim 33, wherein the endometrial cell marker comprises estrogen or progesterone.

30

41. The method of claim 33, wherein the sample is menstrual fluid.

42. The method of claim 33, wherein the subject is not suspected of having endometrial

cancer.

43. The method of claim 33, wherein the subject is not suspected of having endometrial precancer.

5

44. The method of claim 33, wherein the sample is obtained from a subject having or suspecting of having elevated unopposed estrogen levels.

45. The method of claim 33, wherein the sample is obtained from a subject receiving or
10 having received unopposed estrogen treatment.

46. A method for diagnosing endometrial precancer in a subject comprising
obtaining a biological sample of endometrial tissue or cells from a subject,
isolating the endometrial tissue or cells from the sample,
15 contacting the isolated endometrial tissue or cells with an antibody or antigen-binding
fragment thereof that specifically binds PTEN,
determining specific binding between the antibody or antigen-binding fragment
thereof and PTEN in the isolated tissue or cell sample as a diagnosis for endometrial
precancer in the subject.

20

47. The method of claim 46, further comprising
comparing the level of specific binding between the antibody or antigen-binding
fragment thereof and PTEN in the isolated tissue or cell sample, and the level of specific
binding between the antibody or antigen-binding fragment thereof and PTEN in a matched
25 control tissue or cell sample, as a diagnosis for endometrial precancer in the subject.

48. The method of claim 46, wherein the antibody or antigen-binding fragment thereof is
6H2.1 antibody or an antigen-binding fragment thereof.

30 49. The method of claim 46, wherein the sample is menstrual fluid.

50. The method of claim 46, wherein the subject is not suspected of having endometrial
cancer.

51. The method of claim 46, wherein the subject is not suspected of having endometrial precancer.

5 52. The method of claim 46, wherein the sample is obtained from a subject having or suspecting of having elevated unopposed estrogen levels.

53. The method of claim 46, wherein the sample is obtained from a subject receiving or having received unopposed estrogen treatment.

10

54. A kit for diagnosing endometrial precancer in a subject comprising antibodies or antigen-binding fragments thereof that specifically bind PTEN, one or more endometrial cell markers, one or more control molecules, and instructions for the use of the antibodies or antigen-binding fragments thereof, cell markers, and control molecules in the diagnosis of
15 endometrial precancer.

55. The kit of claim 54, wherein the antibodies or antigen-binding fragments thereof are bound to a substrate.

20 56. The kit of claim 54, wherein the antibody or antigen-binding fragment thereof is 6H2.1 antibody or an antigen-binding fragment thereof.

57. The kit of claim 54, wherein the subject is not suspected of having endometrial cancer.

25

58. The kit of claim 54, wherein the subject is not suspected of having endometrial precancer.

59. The kit of claim 54, wherein the sample is obtained from a subject having or
30 suspecting of having elevated unopposed estrogen levels.

60. The kit of claim 54, wherein the sample is obtained from a subject receiving or having received unopposed estrogen treatment.

61. A method for diagnosing endometrial precancer in a subject comprising
obtaining a biological sample of endometrial tissue or cells from a subject,
determining the level of expression of PTEN in the sample, and
5 determining the level of expression of an endometrial cell-associated molecule in the
sample.
62. The method of claim 61, further comprising comparing the levels of expression of
PTEN and one or more endometrial cell-associated molecules to the level of expression of
10 PTEN and one or more endometrial cell-associated molecules in a control sample.
63. The method of claim 61, wherein the endometrial cell-associated molecule is selected
from the group consisting of: estrogen receptor polypeptides and progesterone receptor
polypeptides.
15
64. The method of claim 61, wherein the levels of PTEN and endometrial cell-associated
molecule are determined with nucleic acid amplification methods.
65. The method of claim 61, wherein the levels of expression of PTEN and the
20 endometrial cell-associated molecules are determined with an immunoassay.
66. The method of claim 61, wherein the sample is a body tissue or bodily fluid.
67. The method of claim 61, wherein the sample is menstrual fluid.
25
68. The method of claim 61, wherein the sample is endometrial tissue.
69. The method of claim 61, wherein the subject is not suspected of having endometrial
cancer.
30
70. The method of claim 61, wherein the subject is not suspected of having endometrial
precancer.

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71. The method of claim 61, wherein the sample is obtained from a subject having or suspecting of having elevated unopposed estrogen levels.

72. The method of claim 61, wherein the sample is obtained from a subject receiving or
5 having received unopposed estrogen treatment.

73. A kit for the diagnosis of endometrial precancer comprising oligonucleotides that selectively amplify a nucleic acid sequence that encodes PTEN, oligonucleotides useful for amplifying the nucleic acid sequence that encode one or more endometrial cell-associated
10 molecules, and control nucleic acid primers.

74. The kit of claim 73, wherein the endometrial cell-associated molecule is selected from the group consisting of: estrogen receptor polypeptides or progesterone receptor polypeptides.

15 75. A method for evaluating the effect of candidate pharmacological compounds on endometrial precancer cell phenotype comprising
culturing endometrial tissue or cells,
contacting the cultured endometrial tissue or cells with an antibody or antigen fragment thereof that specifically binds to PTEN,
20 determining a first amount of specific binding of the antibody or antigen fragment thereof with the endometrial tissue or cells,
contacting the cultured endometrial tissue or cells with a candidate pharmacological agent,
contacting the cultured endometrial tissue or cells with the antibody or antigen-
25 binding fragment thereof,
determining a second amount of specific binding of the antibody or antigen-binding fragment thereof, with the cultured endometrial tissue or cells, and
comparing the first and second amounts of specific binding of the antibody or antigen-binding fragment thereof to the tissue or cells, wherein a change in the second amount of
30 specific binding of the antibody or antigen-binding fragment thereof, relative to the first amount of specific binding of the antibody or antigen-binding fragment thereof, indicates the candidate pharmacological compound alters the level of PTEN, wherein a decrease in the relative amount of PTEN indicates the onset of or progression of an endometrial precancer

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cell phenotype, and where an increase in the relative amount of PTEN indicates the regression of an endometrial precancer cell phenotype.

76. The method of claim 75, wherein the antibody or antigen-binding fragment thereof is
5 6H2.1 antibody or an antigen-binding fragment thereof.

77. The method of claim 75, wherein the endometrial tissue or cells are not suspected of having endometrial cancer.

10 78. The method of claim 75, wherein the tissue or cells are not suspected of having endometrial precancer.

79. The method of claim 75, wherein the tissue or cells are from a body tissue or bodily
fluid.

15

80. The method of claim 75, wherein the cells are from menstrual fluid.

81. The method of claim 75, wherein the tissue or cells are endometrial tissue or cells.

20 82. The method of claim 75, wherein the tissue or cells have not been exposed to unopposed estrogen levels.

83. A method for evaluating the effect of candidate pharmacological compounds on endometrial precancer cell phenotype comprising

25 culturing two matched samples of endometrial tissue or cells,

contacting one of the endometrial tissue or cell cultures with a candidate pharmacological agent,

contacting each of the endometrial tissue or cell cultures with an antibody or antigen fragment thereof that specifically binds to PTEN,

30 determining the amount of specific binding of the antibody or antigen fragment thereof of each of the endometrial tissue or cell cultures, and

comparing the amounts of specific binding of the antibody or antigen-binding fragment thereof to the tissue or cell cultures, wherein a difference in the amount of specific

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binding of the antibody or antigen-binding fragment thereof in the culture contacted with the candidate pharmacological agent, relative to the amount of specific binding of the antibody or antigen-binding fragment thereof in the culture not contacted with the candidate pharmacological agent, indicates the candidate pharmacological compound alters the level of PTEN, wherein a decrease in the relative amount of PTEN indicates the onset of or
5 progression of an endometrial precancer cell phenotype, and where an increase in the relative amount of PTEN indicates the regression of an endometrial precancer cell phenotype.

84. The method of claim 83, wherein the antibody or antigen-binding fragment thereof is
10 6H2.1 antibody or an antigen-binding fragment thereof.

85. The method of claim 83, wherein the endometrial tissue or cells are not suspected of having endometrial cancer.

15 86. The method of claim 83, wherein the tissue or cells are not suspected of having endometrial precancer.

87. The method of claim 83, wherein the tissue or cells are from a body tissue or bodily
fluid.
20

88. The method of claim 83, wherein the cells are from menstrual fluid.

89. The method of claim 83, wherein the tissue or cells are endometrial tissue or cells.

25 90. The method of claim 83, wherein the tissue or cells have not been exposed to unopposed estrogen levels.

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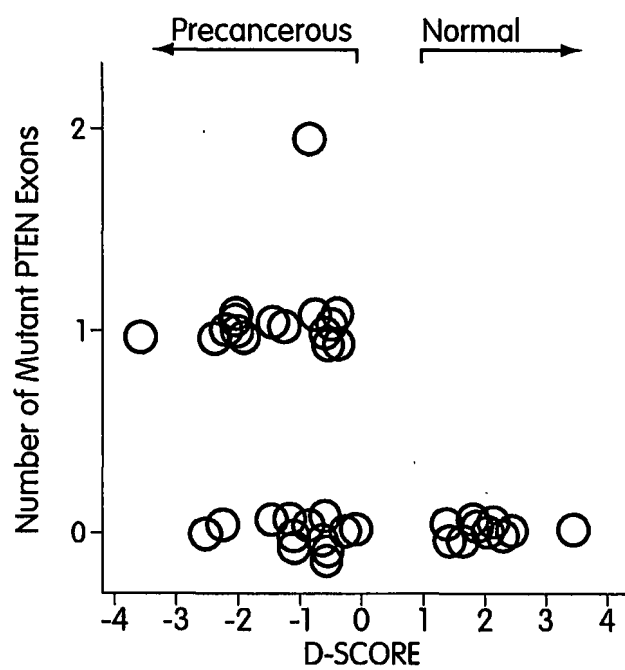


Fig.1

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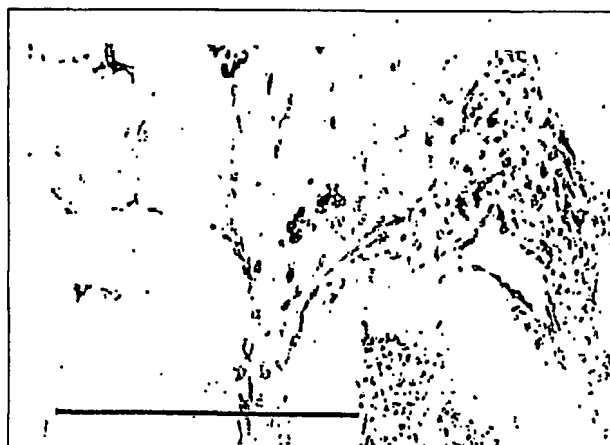


Fig. 2A



Fig. 2B

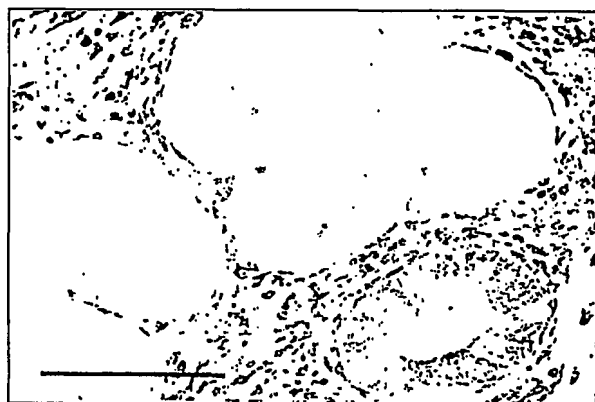


Fig. 2C

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Fig. 2D



Fig. 2E



Fig. 2F

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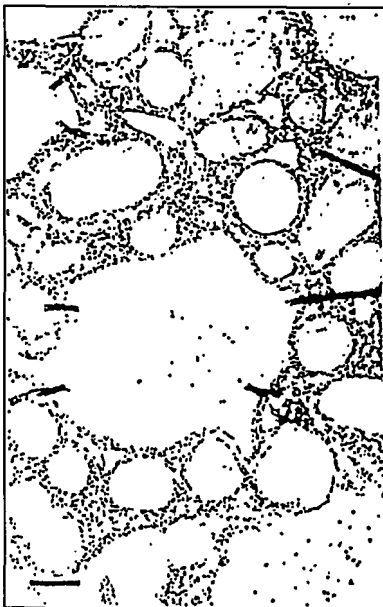


Fig. 3A



Fig. 3B

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Fig. 3C



Fig. 3D

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Fig. 3E



Fig. 3F

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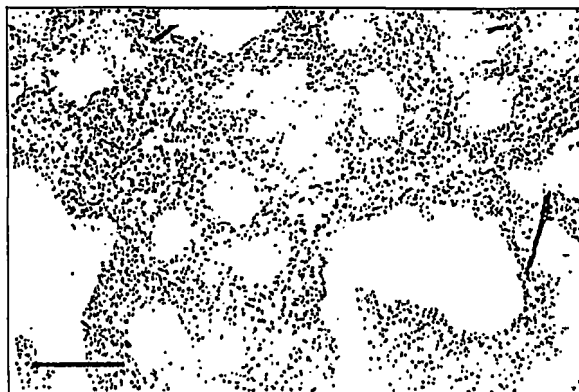


Fig. 3G

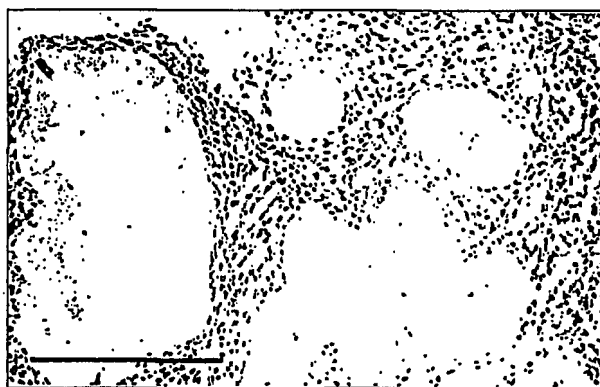


Fig. 3H

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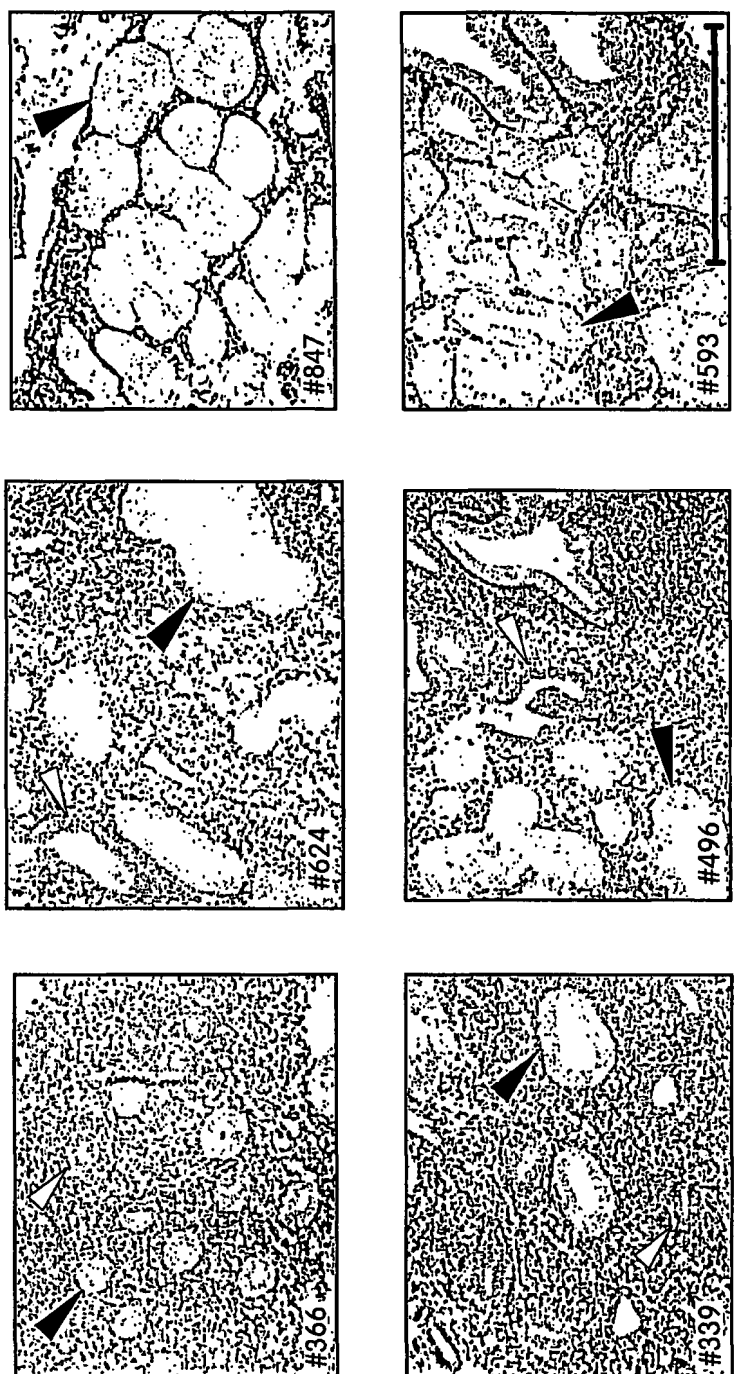


Fig. 4

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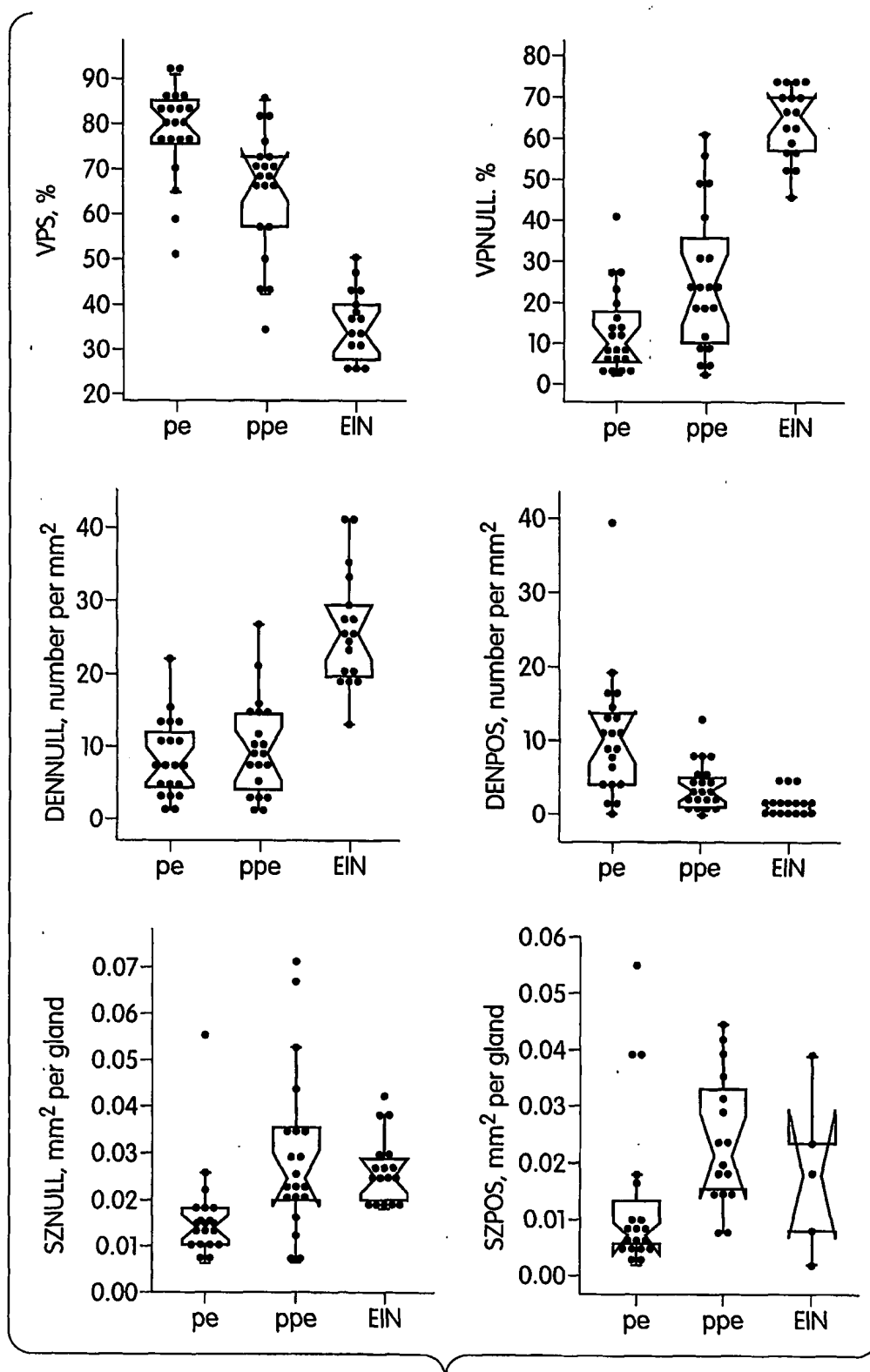


Fig. 5

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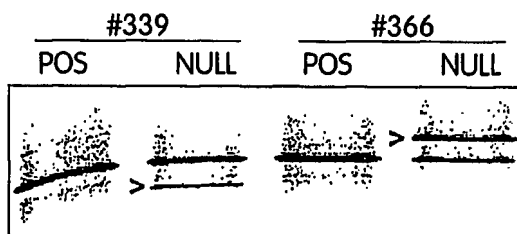


Fig. 6A

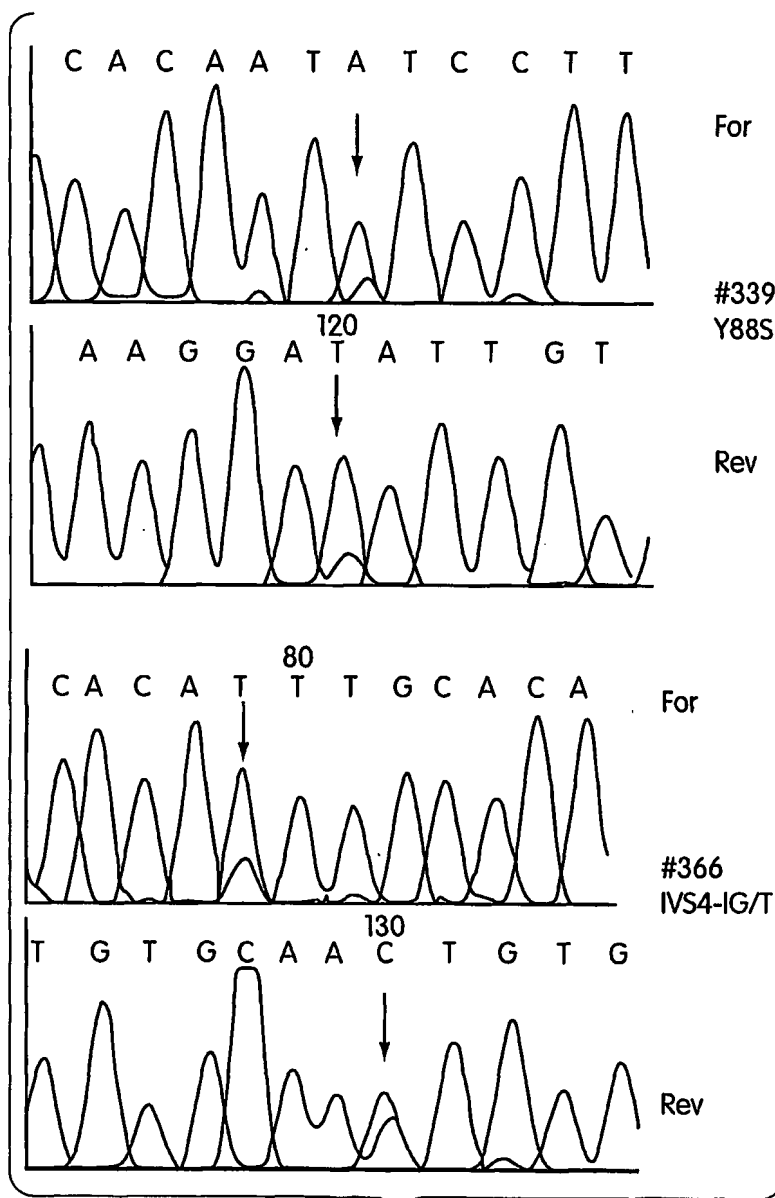


Fig. 6B

-1-

SEQUENCE LISTING

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Ohio State University

<120> Diagnosis of Endometrial Precancers

<130> B0801/7173WO (JRV)

<150> US 60/208,542

<151> 2000-06-01

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<151> 2001-05-08

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/17489

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07K 16/00; C12N 5/02; C12Q 1/68; G01N 33/53, 33/574
US CL : 435/6, 7.1, 7.23, 383, 395; 530/388.8, 389.7

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.23, 383, 395; 530/388.8, 389.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RAMASWAMY, S, et al. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase / Akt pathway. Proc. Natl. Acad. Sci., USA. March 1999, Vol. 96, pages 2110-2115; see entire document.	1-72, and 75-90
Y	KOSHIYAMA, M, et al. Pathology, hormonal aspects, and molecular genetics of the two types of endometrial cancer. The Cancer Journal. November-December 1998, Vol. 11, No. 6, pages 277-283; see the entire document.	20-72, and 75-90
Y	LIN, WM, et al. Loss of heterozygosity and mutational analysis of the PTEN/MMAC1 gene in synchronous endometrial and ovarian carcinomas. Clinical Cancer Research. November 1998, Vol. 4, No. 11, pages 2577-2583; see the entire document.	20-72, and 75-90
Y	RISINGER, JI, et al. PTEN mutation in endometrial cancers is associated with favorable clinical and pathologic characteristics. Clinical Cancer Research. December 1998, Vol. 4, No. 12, pages 3005-3010; see the entire document.	20-72, and 75-90

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

13 August 2001 (13.08.2001)

Date of mailing of the international search report

17 OCT 2001
Authorized officer

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703)305-3230

Stephen L. Rawlings, Ph.D.

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/17489

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P — Y,P	MUTTER, GL, et al. Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. Journal of the National Cancer Institute. 07 June 2000, Vol. 92, No. 11, pages 924-931; see entire document.	20-72 ----- 75-90

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/17489

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/17489

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group 1, claim(s) 1-72, drawn to a method for determining the likelihood of a group of endometrial cells to become cancerous, a method for determining regression, progression, or onset of a condition characterized by abnormal levels of PTEN, a method for monitoring the progression of endometrial precancers, a method for monitoring the regression of endometrial precancers, a method for selecting a treatment for endometrial precancer or endometrial cancer in a subject, a method for diagnosing endometrial precancer in a subject, and a kit comprising an antibody or an antigen-binding fragment thereof.

Group 2, claim(s) 73 and 74, drawn to a kit comprising an oligonucleotide(s).

Group 3, claim(s) 75-90, drawn to a method for evaluating the effect of candidate pharmacological compounds on endometrial precancer cell phenotype.

The inventions listed as Groups 1-3 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of Group 1 is measuring the level of PTEN in a sample of endometrial cells.

The special technical feature of Group 2 is an oligonucleotide(s).

The special technical feature of Group 3 is contacting endometrial cells with a candidate pharmacological agent and measuring the effect of said agent on the cells.

Therefore, Groups 1-3 are not so linked by the same or corresponding special technical feature as to be considered a single general inventive concept. Additionally, PCT Rules 13.1 and 13.2 do not provide for inventions comprising claims drawn to more than one product.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In order for more than one species to be examined, the appropriate additional examination fees must be paid.

Claim 61 is generic to a plurality of disclosed distinct species comprising a method wherein the levels of PTEN and endometrial cell-associated molecule are determined by (a) nucleic acid amplification methods (claim 64) or (b) immunoassays (claim 65).

The species listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons:

The special technical feature of Species (a) is an oligonucleotide.

The special technical feature of Species (b) is an antibody.

Therefore, species (a) and (b) are not so linked by the same or corresponding special technical feature as to be considered a single general inventive concept.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/17489

Continuation of B. FIELDS SEARCHED Item 3:

EMBASE, MEDLINE, BIOSIS: PTEN, MMAC1, cancer, endometrium, progression, diagnosis, prognosis, immunochemistry, anti-PTEN antibody, anti-MMAC1 antibody, RT-PCR